

University of Alberta

Characterization of *unc45b* in zebrafish muscle development

by

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Abstract

The assembly of myosin into higher order structures is dependent upon accessory factors that are often tissue specific. UNC-45 acts as such a molecular chaperone for myosin in the nematode *Caenorhabditis elegans*, in both muscle and non-muscle contexts. Temperature sensitive alleles of *unc-45* cause paralysis due to disorganization of the muscle thick filaments in *C. elegans* while a reduction of maternally contributed *unc-45* results in cytokinetic and polarity defects.

I have identified vertebrate homologues of UNC-45 to assess their requirement for muscle function in these systems. Mammalian and zebrafish *unc45a* are shown to be broadly expressed, with enrichment in the vascular system in zebrafish. In contrast, zebrafish *unc45b* is expressed exclusively in striated muscle tissue, including the somites, heart, pectoral fin muscle and craniofacial muscle. Morpholino-oligonucleotide mediated knockdown of zebrafish *unc45b* results in paralysis and cardiac dysfunction. This paralysis is correlated with a loss of myosin filaments in the sarcomeres of the trunk muscle. Morphants also lack circulation and undergo improper cardiac morphogenesis. Severe cardiac and yolk-sac edema and ventral displacement of several jaw cartilages is also observed when levels of Unc45b are reduced. Overall, this confirms a role for *unc45b* in zebrafish motility consistent with a function in myosin thick filament assembly and stability and uncovers previously unrecognized roles for this gene in the function and morphogenesis of the developing heart and jaw. These results suggest that Unc45b acts as a chaperone that aids in the folding of myosin isoforms required for skeletal, cranial and cardiac muscle contraction.

I have also initiated studies on three strains of mutant zebrafish with characteristics indicative of striated muscle dysfunction. While these mutants do not possess mutations in *unc45b*, they do display phenotypes similar to those caused by a reduction in Unc45b. It is hypothesized that the causative mutations in these animals either affect components of the sarcomere or pathways involved in its formation and organization. Further research on these mutant lines will help to further elucidate molecular components required for the function of striated muscle.

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Table of Contents

1. Introduction	1
1.1 Vertebrate muscle structure and mechanisms of contraction	2
1.1.1 Striated muscle structure	2
1.1.2 Mechanism of muscle contraction	4
1.1.3 Skeletal muscle fiber types	5
1.1.4 Considerations specific to cardiac muscle	6
1.2 Human myopathies	7
1.3 Chaperones and myosin assembly	10
1.3.1 Myosin structure	12
1.3.2 Myosin folding	14
1.4 <i>unc-45</i> in <i>Caenorhabditis elegans</i>	15
1.4.1 <i>unc-45</i> in <i>C. elegans</i> muscle	15
1.4.2 <i>unc-45</i> in the <i>C. elegans</i> embryo	18
1.5 Fungal UCS proteins	20
1.5.1 She4p in <i>Saccharomyces cerevisiae</i>	20
1.5.2 CRO1 in <i>Podospora anserina</i>	21
1.5.3 Rng3p in <i>Schizosaccharomyces pombe</i>	22
1.6 Mammalian <i>unc-45</i>	23
1.7 Zebrafish (<i>Danio rerio</i>) as a model system	24

1.8 Myogenesis in zebrafish.....	26
1.9 Zebrafish heart development	28
1.10 Other known myosins in zebrafish.....	29
1.11 Specific goals of this thesis.....	31
1.13 Summary of this thesis	32
2. Materials and Methods	48
2.1 Zebrafish strains used.....	48
2.2 Zebrafish care.....	49
2.3 Bioinformatic identification of vertebrate <i>unc-45</i> homologues.....	49
2.4 Human Northern Analysis.....	50
2.5 Cloning of zebrafish <i>unc45a</i>	51
2.6 Whole mount <i>in situ</i> hybridization.....	52
2.6.1 Preparation of DIG-labeled RNA probes.....	52
2.6.2 <i>In situ</i> hybridization of mouse embryos	53
2.6.3 <i>In situ</i> hybridization of zebrafish embryos	55
2.7 Developmental RT-PCR.....	56
2.8 Morpholino design and injection	57
2.9 Construction of <i>unc45b::GFP</i> fusion.....	58
2.10 Construction of <i>Myc::unc45b</i> fusion.....	59
2.11 Immunostaining	60

2.11.1 Immunofluorescence.....	60
2.11.2 DAB staining.....	61
2.12 Phalloidin staining.....	61
2.13 Transmission electron microscopy.....	62
2.14 Alcian blue staining.....	62
2.15 Expression of GST::Unc45b in <i>E. coli</i>	63
2.16 Antibody production and purification.....	64
2.17 Testing reactivity and specificity of antisera.....	66
2.17.1 Bacterial and zebrafish lysate.....	66
2.17.2 Western analysis.....	66
2.18 Identification of <i>herzschlag</i> , <i>jam</i> and <i>still heart</i> heterozygotes.....	67
2.19 Sequencing of <i>unc45b</i> from <i>herzschlag</i> and <i>still heart</i> homozygotes	67
2.20 Photography and image processing.....	68
3. Results	70
3.1 Comparison of vertebrate <i>unc-45</i> homologues.....	70
3.2 Expression of mammalian <i>UNC45A</i>	72
3.2.1 Human Northern analysis	73
3.2.2 Mouse <i>in situ</i> hybridization	73
3.3 Expression of <i>unc-45</i> in zebrafish.....	74
3.3.1 <i>In situ</i> hybridization.....	74

3.3.3 RT-PCR	75
3.4 Protein localization (Myc::Unc45b fusion)	76
3.5 Phenotype of <i>unc45b</i> morphants	77
3.6 Myogenic markers in <i>unc45b</i> morphants	79
3.7 Skeletal muscle organization in <i>unc45b</i> morphants	79
3.8 Slow muscle myosin organization in morphants	80
3.9 Cardiac abnormalities in morphants	81
3.9.1 <i>In situ</i> hybridization	81
3.9.2 Immunostaining	82
3.9.3 Improper cardiac morphogenesis and edema	83
3.10 Effects of Unc45b knockdown on jaw structure	84
3.11 Antibody Production and Purification	84
3.12 Phenotypes of mutants	85
3.12.1 <i>herzschlag</i>	86
3.12.2 <i>still heart</i>	86
3.12.3 <i>jam</i>	86
3.13 Mutant muscle structure	87
3.14 Heart structure in mutants	88
3.15 Jaw structure in mutants	89
3.16 <i>unc45b</i> in mutants	89
3.16.1 Sequencing of <i>unc45b</i> cDNA from <i>herzschlag</i> and <i>still heart</i>	89

3.16.2 <i>In situ</i> hybridization.....	90
4. Discussion	138
4.1 Why study Unc45 in zebrafish?.....	138
4.2 <i>unc-45</i> is duplicated in vertebrate genomes.....	139
4.3 Comparison of <i>unc-45</i> phenotypes in <i>C. elegans</i> and zebrafish skeletal muscle. 141	
4.3.1 <i>unc45b</i> expression in the zebrafish embryo.....	141
4.3.2 Phenotype of <i>unc45b</i> morphants	142
4.4 Unc45b in other striated muscle.....	144
4.4.1 Unc45b has a role in zebrafish cardiac development.	144
4.4.2 Requirement of Unc45b in craniofacial development.....	146
4.5 Is Unc45b a molecular myosin chaperone?.....	147
4.6 Potential causative mutations in <i>herzschlag</i> , <i>still heart</i> , and <i>jam</i>	148
4.7 Future Directions.....	150
4.8 Conclusions.....	155
5. Footnotes	157
6. References	158
7. Appendices	175
Appendix I Primer Sequences	175
Appendix II Nucleotide sequence of <i>unc45a</i> cDNA	177

Appendix III Protein sequence of Unc45a..... 180

Appendix IV Nucleotide sequence of *unc45b* cDNA..... 181

Appendix V Protein sequence of Unc45b..... 185

List of Tables

Table 1.1 Known type II myosins in zebrafish striated muscle	33
Table 1.2 Other known myosins in zebrafish	34
Table 3.1 Exon and intron sizes of <i>unc45a</i>	91
Table 3.2 Exon and intron sizes of <i>unc45b</i>	92
Table 3.3 Striated muscle structure and organization.....	93

List of Figures

Figure 1.1 Striated muscle structure and organization	35
Figure 1.2 Transverse tubules and sarcoplasmic reticulum	36
Figure 1.3 Mechanism of muscle contraction	37
Figure 1.4 Cardiac muscle organization	38
Figure 1.5 Mode of action of Hsp70 and Hsp90	39
Figure 1.6 Myosin and thick filaments	40
Figure 1.7 Protein domains of <i>C. elegans</i> UNC-45	41
Figure 1.8 Localization of UNC-45 in <i>C. elegans</i>	42
Figure 1.9 Model of UNC-45 as a myosin co-chaperone	43
Figure 1.10 Model of regulation of UNC-45 levels	44
Figure 1.11 Structure of the UCS proteins	45
Figure 1.12 Myotome organization and muscle specification	46
Figure 1.13 Cardiac specification and structure in zebrafish	47
Figure 3.1 Comparison of human UNC45A with other UCS proteins	94
Figure 3.2 Comparison of zebrafish Unc45 proteins with other UCS proteins	95
Figure 3.3 Protein alignment of the zebrafish Unc45a and Unc45b homologues	96
Figure 3.4 Protein alignment of zebrafish Unc45b and its human, <i>C. elegans</i> and yeast homologues	97
Figure 3.5 Genomic organization of the <i>unc45a</i> and <i>unc45b</i> genes	99
Figure 3.6 Genomic regions containing the <i>unc45a</i> and <i>unc45b</i> genes	100
Figure 3.7 Northern analysis of human <i>UNC45A</i>	101
Figure 3.8 <i>In situ</i> hybridization of mouse embryos	102
Figure 3.9 Expression pattern of <i>unc45a</i>	103
Figure 3.10 Expression pattern of <i>unc45b</i> in striated muscle of the trunk	104

Figure 3.11 <i>unc45b</i> expression in other striated muscle.....	105
Figure 3.12 Expression pattern of <i>unc45b</i> in cardiac mesoderm	106
Figure 3.13 RT-PCR to determine expression profile of <i>unc45b</i> at various developmental stages.....	107
Figure 3.14 Localization of Myc::Unc45b within muscle cells	108
Figure 3.15 Specificity of <i>unc45b</i> MO (1).....	109
Figure 3.16 Comparison of control MO-injected and <i>unc45b</i> MO (1)-injected animals	110
Figure 3.17 Motility of 24 hpf morpholino-injected embryos	111
Figure 3.18 Myogenic determination is not altered in morphants.....	112
Figure 3.19 DIC photography of trunk muscle.....	113
Figure 3.20 Actin filaments at 24 hpf and 48 hpf.....	114
Figure 3.21 Ultrastructure of trunk muscle at 48 hpf	115
Figure 3.22 Immunostaining with anti-myosin antibodies	116
Figure 3.23 Expression pattern of cardiac markers	117
Figure 3.24 Protein localization of cardiac markers.....	118
Figure 3.25 Cardiac abnormalities in <i>unc45b</i> morphants	119
Figure 3.26 <i>unc45b</i> morphants display dysmorphic jaw structure at 5 dpf.....	120
Figure 3.27 Expression of GST::Unc45b protein and antibody production	121
Figure 3.28 Comparison of wildtype and <i>herzschlag</i> animals.....	123
Figure 3.29 Comparison of wildtype and <i>still heart</i> animals.....	124
Figure 3.30 Comparison of wildtype and <i>jam</i> animals.....	125
Figure 3.31 DIC photography of trunk muscle in mutant embryos.....	126
Figure 3.32 Immunostaining of <i>herzschlag</i> embryos with anti-myosin antibodies	127
Figure 3.33 Immunostaining of <i>still heart</i> embryos with anti-myosin antibodies	128
Figure 3.34 Immunostaining of <i>jam</i> embryos with anti-myosin antibodies	129

Figure 3.35 Analysis of cardiogenesis of <i>herzschlag</i> embryos using cardiac <i>in situ</i> markers and antibodies	130
Figure 3.36 Analysis of cardiogenesis of <i>still heart</i> embryos using cardiac <i>in situ</i> markers and antibodies	131
Figure 3.37 Analysis of cardiogenesis of <i>jam</i> embryos using cardiac <i>in situ</i> markers and antibodies	132
Figure 3.38 <i>herzschlag</i> mutant larvae display dysmorphic jaw structure at 5 dpf	133
Figure 3.39 <i>still heart</i> mutant larvae display dysmorphic jaw structure at 5 dpf	134
Figure 3.40 <i>jam</i> mutant larvae do not display jaw structure abnormalities at 5 dpf	135
Figure 3.41 Expression of <i>unc45b</i> in <i>herzschlag</i> embryos	136
Figure 3.42 Expression of <i>unc45b</i> in <i>still heart</i> embryos	137

List of Symbols, Nomenclature, and Abbreviations

This text follows the proper gene/protein nomenclature for each organism discussed. Gene names are italicized. Protein names are capitalized and not italicized.

Abbreviations/Symbols	Description
aa	amino acid
BAC	bacterial artificial chromosome
BLAST	Basic Local Alignment Search Tool
bp	base pairs
Ce	<i>Caenorhabditis elegans</i>
CMV	cauliflower mosaic virus
DCM	dilated cardiomyopathy
DEPC	diethyl pyrocarbonate
DIC	differential interference contrast
DIG	digoxigenin
dpc	days post coitus
dpf	days post fertilization
Dr	<i>Danio rerio</i>
ELC	essential light chain
EST	expressed sequence tag
GC	general cell
GFP	green fluorescent protein
GST	glutathione S-transferase
HCM	hypertrophic cardiomyopathy
hpf	hours post fertilization

h	hour
Hs	<i>Homo sapiens</i>
Hsp	heat shock protein
Hz	hertz
kbp	kilobase pairs
MHC	myosin heavy chain
min	minute
MO	morpholino oligonucleotide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PGC	primordial germ cell
RACE	rapid amplification of cDNA ends
RLC	regulatory light chain
RNAi	RNA interference
rpm	revolutions per minute
RT	reverse transcriptase
SDS	sodium dodecyl sulphate
SSC	sodium chloride sodium citrate
Sc	<i>Saccharomyces cerevisiae</i>
sec	seconds
SM	striated muscle
SMAP	smooth muscle-associated protein

TEM	transmission electron microscopy
TPR	tetratricopeptide repeats
ts	temperature sensitive
unc	uncoordinated
UCS	UNC-45/CRO1/She4p domain; also a family of proteins containing this domain.
UTR	untranslated region

1. Introduction

Striated muscle contractility is essential for the survival of most animals, being required for both mobility and cardiac function. Because striated muscle structure and organization are incredibly complicated it is not surprising that many genetic causes have been implicated in skeletal and cardiac myopathies. One component that is frequently involved in both skeletal and cardiomyopathy is myosin, a large motor protein in which the precise folding of the globular head domain is necessary for proper function and thereby contractility in the context of skeletal and cardiac muscle. The nature of this head domain requires the activity of molecular chaperones to aid in it adopting its functional conformation. UNC-45 is one potential chaperone that may be involved in the folding of myosin.

unc-45 was first discovered in *C. elegans* as being essential for muscle function/motility and embryo development/cytokinesis. Several proteins that are also involved in myosin-related processes share a domain with this protein known as the UCS domain. Proteins containing a UCS domain exist in almost all species of metazoans examined, from fungi to mammals. However vertebrates, including zebrafish, possess two functionally distinct *unc-45* homologues termed *unc45a* and *unc45b*. *unc45a* is widely expressed whereas expression of the *unc-45b* homologue is restricted to striated muscle. This thesis focuses on the use of molecular techniques to demonstrate the importance of *unc45b* in the development and function of these striated muscle tissues in the zebrafish, *Danio rerio*.

1.1 Vertebrate muscle structure and mechanisms of contraction

There are three types of muscle: skeletal, cardiac and smooth. Skeletal muscle makes up approximately 50% of human body mass, is striated in appearance and is multinucleate. It is this type of muscle that allows the body to perform voluntary motor activities. Like skeletal muscle, cardiac muscle is striated but cardiac muscle cells contain only one nucleus and, as expected as it is found only in the heart, its contractile activity is involuntary. In contrast to the other two types of muscle, smooth muscle is not striated and, similar to cardiac muscle, it is mononucleate. Smooth muscle is found in the walls of hollow organs in humans and its contraction is responsible for the movement of blood through the vessels, food through the digestive tract and waste through the urinary system. These actions, like heart pumping, are required to sustain life and are therefore controlled by the autonomic nervous system instead of the somatic nervous system and are not subject to control by the individual.

1.1.1 Striated muscle structure

Muscle fibers, the main component of muscle cells, are composed of myofibrils that can span the entire length of a muscle cells. These myofibrils are a series of sarcomeres, with each sarcomere being composed of over 40 proteins (Laing and Nowak, 2005). The repetition of these sarcomeric units leads to the striation of muscle and this regular organization is critical for function. Myofibrils form bundles that are anchored to the plasma membrane of the cell by associating with each other through desmin and intermediate filaments (Figure 1.1A-C) (Granger and Lazarides, 1979).

The sarcomeres that make up the myofibrils are comprised mainly of actin and myosin. Thin filaments are composed mostly of G-actin (globular actin) subunits that form polymers organized as double stranded helixes known then as F-actin (filamentous actin) (Clark et al., 2002). At regular intervals a troponin complex consisting of three proteins (one of which binds actin, one of which binds tropomyosin and one of which binds calcium) and tropomyosin interact with these actin filaments (Figure 1.1E). When muscle is relaxed, tropomyosin hides the myosin cross-bridge binding sites on actin, thereby physically preventing the interaction between actin and the thick filaments (Murakami et al., 2007). Through a variety of proteins, including α -actinin, thin filaments are anchored to Z lines, regions that demark the edges of individual sarcomeres (Stromer, 1995). Thick filaments are made up of myosin molecules, associated into filaments through their tail domains (Figure 1.1D, expanded upon below) (Craig and Woodhead, 2006). The area of the sarcomere consisting of the thick filaments is commonly referred to as the A band (versus the I band, the region composed only of thin filaments, without thick filament overlap). Upon completion of myofibril assembly the H zone (the center of the sarcomere where only thick filaments are found as the thin filaments do not reach this far) is bisected by structures referred to as M lines, thought to be anchoring sites for thick filaments (Agarkova and Perriard, 2005; Knappeis and Carlsen, 1968). These M lines are then anchored to the plasma membrane via desmin and vimetin, components of intermediate filaments (Granger and Lazarides, 1979).

There are a couple of other noteworthy proteins involved in sarcomere assembly. Titin (the largest protein yet described) is an elastic molecule that reaches from the thick filament to the Z line (Wang et al., 1979). It interacts with myosin through myosin-

binding protein C and is thought to keep the thick filaments centered in the sarcomere and to act as a template for sarcomere assembly (Horowitz et al., 1986; Labeit et al., 1990). Nebulin, on the other hand, associates with actin and stretches the entire length of the thin filament (Wang and Williamson, 1980). Its length is thought to regulate the length of these actin filaments (McElhinny et al., 2005).

1.1.2 Mechanism of muscle contraction

Muscle fibers are invaded by extensions of the plasma membrane known as transverse tubules (T-tubules). When acetylcholine is released at neuromuscular junctions an action potential is created which induces permeability changes in T-tubules. This triggers calcium release into the cytosol from the sarcoplasmic reticulum, a modified endoplasmic reticulum that surrounds each myofibril (Peachey, 1965) (Figure 1.2). Calcium then binds to a member of the troponin complex, altering the conformation of the troponin-tropomyosin complex thereby uncovering the sites of actin that are able to interact with myosin (Drabikowski et al., 1968; Murakami et al., 2007; Perry, 2001).

The myosin head domain (the structure of myosin is discussed further below) has an actin-binding site and a catalytic ATPase domain. Before myosin attaches to actin it hydrolyzes a molecule of ATP to ADP and P_i which remain bound to the myosin (Yu et al., 1970). When calcium enters a muscle cell and myosin cross-bridges are able to interact with actin. The myosin molecule converts the chemical energy from the aforementioned ATP hydrolysis into mechanical energy and bends creating a power stroke, after which ADP and P_i are released (Tyska and Warshaw, 2002). The actin and myosin molecules remain in contact until myosin binds a new ATP molecule that can

then be hydrolyzed, reinitiating the power stroke cycle. These power strokes physically pull the thin filaments towards the center of the sarcomere, resulting in contraction of the muscle (Hansen and Huxley, 1954). In the absence of stimulation relaxation is able to occur through the active return of calcium to the sarcoplasmic reticulum. This is achieved through the involvement of the sarcoplasmic reticulum's Ca^{2+} -ATP-ase pump. The lack of cytoplasmic calcium allows the troponin-tropomyosin complex to return to its blocking position so actin and myosin are no longer able to interact (Figure 1.3).

1.1.3 Skeletal muscle fiber types

Vertebrate muscle fibers can be classified as slow (Type I - slow oxidative) or fast (Type IIa - fast-oxidative and Type IIb and Type IIc/x - fast-glycolytic) depending on the presence of certain myosin isoforms that differ in speed of ATP hydrolysis (Fox, 2004; Sherwood, 2001). In zebrafish slow muscles are mononucleate and contain many mitochondria, due to higher reliance on oxidative phosphorylation. This type of muscle has higher myoglobin content which gives them a red color. Fast fibers split ATP more rapidly and allow for faster contraction, hence the term fast twitch muscles. The multinucleate cells of Type IIb and Type IIc/x fibers contain few mitochondria relative to slow fibers as they are specialized to undergo glycolysis that requires less oxygen than oxidative phosphorylation. Due to the lower oxygen requirement they contain little myoglobin, giving them a white appearance. Type IIa fibers have qualities of both of the other Type I and Type IIb fibers. They have high ATPase activity and employ oxidative phosphorylation to produce ATP for energy. However they do rely partially on

glycolysis to maintain ATP production at levels that are required due to the rapid ATP splitting.

1.1.4 Considerations specific to cardiac muscle

Cardiac muscle is also striated and functions nearly identically to slow twitch skeletal muscle. In contrast to skeletal muscle cells however, cardiac cells are shorter and connected to each other at their ends through gap junctions (Figure 1.4). The gap junctions allow myocardial cells to act in unison when they are electrically stimulated as the impulse is then spread to all cells simultaneously. A region of the atrium that functions as a pacemaker, called the sinoatrial node, spontaneously creates an action potential that results in the release of calcium into the cytoplasm which results in sarcomere contraction in a manner identical to that in skeletal muscle. Calcium is then actively transported out of the cells to return the cells to a relaxed state. The ventricle contracts slightly later than the atrium because the stimulus cannot travel directly from the atrium to the ventricle. The stimulus therefore must travel through a group of specialized conducting cells called the atrioventricular node resulting in a slight time lapse. It is this lapse that ensures that the atria have expelled the blood into the ventricles before the ventricles contract. This results in the characteristic "lub/dub" sound of a human heartbeat.

1.2 Human myopathies

Myopathy is a general term referring to a devastating class of human diseases that result in muscle weakness or paralysis due to muscle degeneration. It is easy to imagine due to the sophisticated nature of muscle function that there are numerous pathways that when altered can lead to these conditions. Many of these diseases, though not all, have genetic causes. Whatever the cause for the diseases they affect the skeletal muscle system (skeletal myopathies) and/or the heart (cardiomyopathies). Obviously both skeletal and cardio-myopathies can have severe consequences on the health of the affected individual.

There are many classes of myopathies, classified based on their underlying causes. Neuromyopathies are a class of disorders that affect both the nervous system and the musculature. These develop when the neurons associated with muscular tissue either do not develop or progressively degenerate. Amyotrophic lateral sclerosis (ALS or Lou Gehrig's disease) is one of the most well known neuromyopathies though its exact cause is yet unknown (Jackson and Bryan, 1998; Shoesmith and Strong, 2006). Because the motor neurons no longer send signals to the skeletal muscle in ALS patients the muscle atrophies eventually resulting in paralysis and death, usually due to respiratory failure. Mitochondrial myopathies are another class of myopathies, resultant, as the name implies, from mitochondrial dysfunction (DiMauro, 2006; DiMauro and Gurgel-Giannetti, 2005). Because muscle requires large amounts of energy, it is easy to see how damage to the mitochondria could affect muscle function. Kearns-Sayre syndrome is one such disease. This disease can result in muscle weakness, paralysis of certain eye muscles and cardiomyopathies (Maceluch and Niedziela, 2006). Inflammatory

myopathies occur when the body attacks its own muscle tissue in an autoimmune response. The main inflammatory myopathies are dermatomyositis, where the small blood vessels that supply the skin and muscle are damaged and polymyositis, where the muscle fibers are directly attacked (Callen and Wortmann, 2006; Dalakas, 2006; Dalakas and Hohlfeld, 2003). Luckily both of these diseases are usually easily treated with drugs that suppress the body's immune system (Lundberg and Alexanderson, 2007).

Additionally myopathies can also result from metabolic or endocrine disorders.

Metabolic myopathies are usually caused by defects in enzymes that control the breakdown of fuel leading to muscle weakness and heart problems (Bartram et al., 1995; Fukuda et al., 2007). Endocrine-related myopathies can be caused by dysfunction of the adrenal, thyroid, parathyroid or pituitary systems, with each leading to its own set of symptoms (Heckmann et al., 2000; Hekimsoy and Oktem, 2005). The underlying etiologies of many of the above diseases are unknown. They may have genetic causes or occur spontaneously due to environmental factors such as viral infection or drug exposure.

The last class of myopathies results from defects in structural proteins. Human skeletal myopathies have been associated with mutations in 20 of the sarcomeric proteins (Bonnemann and Laing, 2004; Laing and Nowak, 2005). Many of these genes have numerous mutations associated with them and these different mutations can lead to distinct diseases, each with their own characteristics. It is apparent that these skeletal myopathies cannot be easily grouped as: their age of onset varies from *in utero* to adulthood, the severity of these disorders can range from mild to early lethal, they display

diverse gross and subcellular phenotypes and can follow a dominant and/or recessive mode of inheritance.

Though cardiomyopathies can occur for extrinsic reasons such as high blood pressure, diabetes, alcohol and drug abuse or viral infection, genetics plays a large role in the development of heart disorders. Similar to human skeletal myopathies, cardiomyopathies are frequently caused by problems with components of the sarcomere. Numerous mutations have been identified in sarcomeric proteins that are associated with dilated cardiomyopathies (DCM) and familial hypertrophic cardiomyopathies (HCM) (Bashyam et al., 2003; Morita et al., 2005; Redwood et al., 1999). DCM results in enlargement of the heart and poor pumping with the left ventricle being especially affected; familial hypertrophic cardiomyopathy (HCM) results in thickening of the heart muscle that can impair blood flow. It is thought that the cardiac cells must be able to detect impairments in contractility in DCM and HCM and, in an attempt to compensate for these defects, initiate morphological and functional alterations (Chang and Potter, 2005; Tardiff, 2005).

Occasionally mutations can cause both skeletal myopathies and cardiomyopathies. A single mutation in the slow beta cardiac myosin, *MYH7*, which encodes a molecular motor, is one such case. This would be expected as this gene is expressed in slow skeletal muscle fibers and the heart (Darin et al., 2007; Fananapazir et al., 1993; Laing and Nowak, 2005). What is unusual about mutations in *MYH7* is that, although it is expressed in both skeletal and cardiac muscle, some mutations cause only skeletal myopathies or cardiomyopathies, not both. Certain mutations only affect distal muscles despite its expression in all slow skeletal muscles while others result in

myopathy associated with myosin storage defects (Jandreski et al., 1987; Meredith et al., 2004). All of the mutations that cause the distal myopathy reside in the myosin tail region and are predicted to impede the formation of a coiled coil (Barohn et al., 1994). Certain cardiomyopathy-associated mutations are also in this region though they are not predicted to affect myosin assembly to the extent that the distal myopathy-associated mutations are (Blair et al., 2002). In spite of the fact that the mutations that cause distal myopathy should affect assembly more severely than those that cause cardiomyopathy, patients with distal myopathy rarely have heart problems. The reasons behind this enigma are unknown.

Due to the number of components involved in striated muscle function and complexity of these disorders, the molecular causes of myopathies resulting from defective sarcomeric components can be difficult to diagnose clinically. Most disorders are caused by *de novo* mutations so linkage analysis is not possible. Furthermore many structural genes are large and undergo alternative splicing so sequencing is not usually an option. Regardless of whether or not disorders are easily identified the prevalence of these types of mutations and their impact demonstrate the importance of sarcomeric function in human health.

1.3 Chaperones and myosin assembly

In order to be functional, proteins must go through a process of folding to adopt their correct conformation. Frequently additional proteins called chaperones assist protein folding. Many human diseases, including some myopathies, are caused when

proper protein structure is not attained (Barral et al., 2004; Gregersen, 2006; Macario and Conway de Macario, 2005). Two common chaperones, Hsp90 and Hsp70, named based on their respective molecular weights, aid in the folding in numerous proteins though the exact mechanisms by which they achieve this remain unknown. Hsp70 associates with hydrophobic protein segments in an ATP-dependent manner in order to fold nascent peptides, prevent aggregation or refold aggregated proteins (Bukau et al., 2006; Mayer and Bukau, 2005; Young et al., 2003). ATP hydrolysis closes the substrate-binding cavity of Hsp70 so that the client protein can only be released once ADP and Pi are released and a new ATP molecule is bound (Figure 1.5A). Following interaction with Hsp70, partially folded substrates may be transferred to other chaperones such as Hsp90. In contrast to Hsp70, Hsp90 does not usually interact with newly formed proteins (Nathan et al., 1997). Instead it usually acts on proteins in a late stage of folding to help them achieve their mature state or to prevent aggregation (Pearl and Prodromou, 2006; Picard, 2002; Young et al., 2003; Young et al., 2001). Alternatively, since a large proportion of Hsp90 targets are involved in signaling, it is thought that Hsp90 might keep molecules inactive until they receive their respective signals followed by release by Hsp90 and completion of folding or it might act following the appropriate signal to aid in maturation. Hsp90 dimerizes through its C-terminal domain and the conformation changes that the dimer undergoes are dependent on ATP hydrolysis, similar to Hsp70. In this case, Hsp90 binds the client protein when Hsp90 is in its nucleotide-free state. Binding of ATP closes the clamp, trapping the substrate until the ATP molecule is hydrolyzed, after which the client protein and ADP and Pi are released (Figure 1.5B).

Selection of client proteins is often reliant on the association of Hsp70 or Hsp90 with co-chaperones or adaptor proteins. These co-chaperones are a diverse group of proteins. Some may have chaperone activity themselves, some regulate the ATPase activity of Hsp90 and some link the client protein and the chaperone. Many co-chaperones are known to contain TPR (tetra-tricopeptide repeat) domains though the mode of action of most co-chaperones is not yet known (Caplan, 2003).

1.3.1 Myosin structure

There are 18 distinct classes of myosins (Berg et al., 2001; Foth et al., 2006). Myosins are identified based on the presence of the 80 kD motor domain that hydrolyzes ATP and is responsible for movement along actin filaments. Some of these are specific to the class of organism they are found in while others are found in almost all eukaryotes. So-called conventional myosin molecules (type II myosins) are composed of two identical myosin heavy chains (MHC), each containing an N-terminal globular head domain, a central neck domain and a C-terminal tail domain that associates with its homodimeric partner in a α -helical coiled-coil fashion. An essential light chain (ELC) and a regulatory light chain (RLC) wrap around the neck domain of each heavy chain to form one complete myosin molecule (Figure 1.6A). Both the ELC and RLC modulate the interaction between actin and myosin (Lowey et al., 1993; Stepkowski, 1995). In striated muscle the ELC is able to bind actin thereby affecting the interaction of actin and myosin but controversy exists as to whether it strengthens or weakens this interaction (Hernandez et al., 2007; Timson, 2003). In the presence of calcium, the RLC is

phosphorylated by myosin light chain kinase. This phosphorylation is thought to increase force production or maintenance (Sweeney et al., 1993; Szczesna, 2003).

Myosin in striated muscle forms filaments via interactions between of hundreds of myosin molecules (Figure 1.6B) (Craig and Woodhead, 2006). At the center of these filaments the interaction is antiparallel creating a region that is free of myosin heads. It is at this bare zone that M-line proteins bind to connect the thick filaments of the sarcomere. The distal regions of the filament are formed by parallel interactions. The tails interact with each other to form filaments based on interactions between positively and negatively charged regions. The myosin heads are quasi-helically arranged and ordered at 14.5 nm, with the turn of each helix spaced at 43 nm (Huxley and Brown, 1967; Stewart and Kensler, 1986; Wray et al., 1975). Each of these distances are determined by regions of particularly strong attraction between tails, staggered by 98 and 294 amino acids, respectively (McLachlan and Karn, 1983). Finally, at the C-terminal end of each myosin molecule is a region of coiled-coil with properties that differ from the rest of the tail that is thought to be involved in the initiation of assembly of filaments (Hoppe et al., 2003; Nakasawa et al., 2005).

While most invertebrate thick filaments contain a paramyosin core that serves to strengthen the filament, vertebrate thick filaments do not contain this molecule. They do interact with non-myosin proteins however. Most notably, titin, consisting of immunoglobulin and fibronectin-like domains arranged in repeat patterns of 43 nm that are thought to determine the locations of myosin-binding proteins, particularly myosin-binding protein C (Clark et al., 2002; Tskhovrebova and Trinick, 2003). It is thought that myosin-binding protein C may be involved in sarcomere assembly by linking titin and

myosin and, at least in cardiac muscle, may be involved in the control of contraction (Granzier and Campbell, 2006).

1.3.2 Myosin folding

Because the myosin head is a globular domain, its proper folding relies heavily on the activity of chaperones. In the absence of muscle-specific factors the head of muscle myosin is unable to adopt its proper conformation (Chow et al., 2002; Srikakulam and Winkelmann, 1999). Candidates for some of these factors have arisen from biochemical analysis. The chaperonin-containing TCP-1 (CCT) interacts with the myosin heavy chain but is not sufficient for myosin to form a functional motor domain when expressed in a rabbit reticulocyte system (Srikakulam and Winkelmann, 1999). Hydrophobic interactions are favored at physiological temperature (30°C) resulting in aggregation of the myosin head or interaction of the head with other cytosolic components (Chow et al., 2002). However at 0°C this domain is able to spontaneously fold in a chaperone-independent fashion but this folding pathway is too slow to be of physiological relevance. More recently Hsp90 and Hsp70 have been implicated in the initial folding of myosin. In the process of forming myofibrils, myosin is first found in globular foci, then in short filamentous structure and finally in striated myosin filaments. Hsp90 and Hsp70 are associated with the myosin folding intermediates, the globular foci and short filaments but not with the striated myofibrils. The myosin motor domains in these structures are only partially folded and remain trapped in this conformation if Hsp90 is unable to dissociate, therefore other uncharacterized factors are likely necessary for myosin to reach its mature state (Srikakulam and Winkelmann, 2004).

1.4 *unc-45* in *Caenorhabditis elegans*

Genetic approaches have considerable advantages in the identification of novel regulators of cellular and developmental processes. The nematode *Caenorhabditis elegans* has been useful in understanding muscle development as many genes first identified via mutations affecting *C. elegans* muscle structure and function have subsequently been shown to have homologues in vertebrate muscle.

1.4.1 *unc-45* in *C. elegans* muscle

One of the first mutations reported to affect *C. elegans* muscle structure was *unc-45* (Epstein and Thomson, 1974). Temperature sensitive (*ts*) alleles of *unc-45* result in paralysis (known as an *unc* or uncoordinated phenotype) at the restrictive temperature due to the disorganization of the thick filament (Barral et al., 1998; Epstein and Thomson, 1974) while null alleles are lethal and result in arrest at the twofold stage and lack all movement and pharyngeal pumping (Venolia and Waterston, 1990). *unc-45*-null embryos lack zygotic *unc-45* expression but do retain some UNC-45 function that is provided maternally.

UNC-45 is a 961 amino acid protein in *C. elegans* consisting of three distinct domains (Figure 1.7) (Barral et al., 1998; Venolia et al., 1999). The amino end of the protein contains three TPR (tetratricopeptide repeat) motifs resembling the TPR domain of Hop (Hsp70/Hsp90-organizing protein) and of protein phosphatase which also interacts with Hsp70 and Hsp90 (Barral et al., 2002). These repeats are thought to be involved in modulating protein-protein interactions. The central region of UNC-45 does not show similarity to any proteins outside of the UNC-45 family. Finally,

approximately the last third of the protein forms the UCS (UNC-45/CRO1/She4p) domain that shows similarity to proteins found in the fungal kingdom (see section 1.5 below).

GFP fusions show this gene is expressed in all *C. elegans* muscle cells (body wall muscle, pharyngeal muscle, sex-specific muscles and anal muscles) and co-localizes with the major myosin heavy chain component (MHC B) of the thick filaments of body wall muscle, but not the minor muscle myosin isoform, MHC A, with its localization being dependent on the localization of MHC B, specifically a region of the myosin rod domain (Figure 1.8A-E) (Ao and Pilgrim, 2000; Venolia et al., 1999). In *unc-45 (ts)* animals, the amount of MHC B present is decreased and the thick filaments no longer display the characteristic localization of MHC A to the central region and MHC B to the distal regions. Instead the localization of each becomes scrambled with each being present across the entire length of the filament (Barral et al., 1998). Consistent with an interaction only between MHC B and UNC-45 in *C. elegans*, if MHC A is overexpressed, UNC-45 is dispensable for mobility. In this situation the thick filaments that assemble are shorter than normal and are composed entirely of MHC A (Ao and Pilgrim, 2000).

Cell culture studies have shown that UNC-45 interacts with both myosin and Hsp90 and plays a role in preventing thermal aggregation of myosin (Barral et al., 2002). The TPR domain is required for the preferential interaction between UNC-45 and Hsp90 but not between UNC-45 and Hsp70 suggesting that the Hsp70-interacting region lies outside of this domain. The chaperone interactions are only apparent at elevated temperatures that enhance hydrophobic interactions. This is commonly observed for associations between chaperones and co-chaperones (Smith et al., 1990). UNC-45,

outside of the TPR domain, is also able to bind the head domain of myosin directly and prevents its aggregation indicative of a role for UNC-45 as a chaperone and/or a co-chaperone with Hsp90 (Figure 1.9).

Recently it has been shown that levels of UNC-45 must be strictly regulated, a process that is accomplished through ubiquitin-mediated degradation (Hoppe et al., 2004). Ubiquitin is a small polypeptide that functions to target proteins to the proteasome for degradation. E3 and E4 proteins are responsible for adding ubiquitin moieties to proteins, forming polyubiquitin chains that are then recognized by proteasome receptors or by adaptor proteins which target the modified protein for degradation (Kerscher et al., 2006). E3 enzymes are known to often interact with the chaperones Hsp70 and Hsp90 (Connell et al., 2001; Imai et al., 2002; Pickart, 2004). A E3/E4 multiubiquitylation complex composed of CHN-1, UFD-2 and CDC-48 regulates UNC-45 levels and is necessary for the appropriate degradation of UNC-45 during adult stages (Hoppe et al., 2004; Janiesch et al., 2007). This data is supported by the appearance of an interaction between UNC-45 and UFD-2 (Kachur et al., 2004). It does not seem that this degradation is as prevalent during larval stages as UNC-45 levels remain high at this time. Although *chn-1* mutations alone do not affect the phenotype of wildtype worms, they are able suppress the phenotype of *unc-45* temperature sensitive animals. However when *unc-45* is overexpressed sarcomeric defects are observed in *chn-1* worms. This implies that additional UNC-45 is toxic to the process of muscle assembly (Figure 1.10). Increased levels of UNC-45, even in wildtype background, also result in defects in motility and thick filament assembly, though not to the same extent as loss of function (Landsverk et al., 2007). In this case MHC A and B levels are reduced to approximately

70% of that of wildtype, a defect that is rescued by partial suppression of the proteasome. Interestingly the human homologue of CDC-48, p97 which has been implicated in adult-onset muscle-wasting myopathy, is able to functionally rescue loss of CDC-48 in *C. elegans* muscle (Janiesch et al., 2007; Watts et al., 2004). Human UNC45B appears to follow the same pattern of ubiquitin-mediated degradation as it is destabilized by UFD2A and stabilized by proteasome inhibitors and by alleles of p97 linked to human myopathies. These results show that in the absence of or in the presence of excess UNC-45, myosin that is not in its mature conformation accumulates which is then degraded by the proteasome and that when this process is impeded, sarcomeric defects are observed which can then lead to myopathies.

1.4.2 *unc-45* in the *C. elegans* embryo

UNC-45 does not only interact with muscle myosin in *C. elegans*. A yeast two-hybrid screen also demonstrated interaction between UNC-45 and HUM-2, a class V myosin, although the link between HUM-2 and UNC-45 function is unclear as null alleles of *hum-2* have no apparent phenotype (Baker and Titus, 1997; Kachur et al., 2004). It is tempting to predict that UNC-45 is acting as a type V myosin chaperone, in addition to its apparent role as a type II myosin chaperone. Another connection between UNC-45 and another type II myosin also came from yeast two-hybrid analysis. UNC-45 was also shown to interact with NMY-2, a non-muscle myosin required for cytokinesis and polarity establishment in *C. elegans* embryogenesis (Guo and Kemphues, 1996; Kachur et al., 2004; Shelton et al., 1999).

In *C. elegans* UNC-45 mRNA and protein is contributed through the maternal germline and is present at the cell cortex of all cells of the early embryo (Kachur et al., 2004). If the maternal contribution of UNC-45 is reduced, severe cytokinetic defects are observed in the early embryo (Kachur et al., 2004). This was determined using embryos heteroallelic for a temperature sensitive and a null allele of *unc-45*. In these embryos, cytokinesis is attempted but rarely completed. Cleavage furrows begin to form but following failure to complete cytokinesis, the furrows regress. However karyokinesis is unaffected, resulting in single cells with multiple nuclei. In animals completely lacking UNC-45 function, achieved through use of RNA interference, more severe problems are observed (Kachur et al., submitted). Cytokinesis defects are still observed but these animals also fail to establish the correct anterior posterior asymmetry, display altered polar body extrusion and fail to undergo germline cellularization, processes that also rely on NMY-2 function. UNC-45 and NMY-2 co-localize at the cell cortex of *C. elegans* embryos with the localization of UNC-45 being dependent on NMY-2 localization (Figure 1.8F). However in *unc-45 (RNAi)* animals, instead of NMY-2 localizing to distinct contractile foci, it is found throughout the cortex indicating that the specific localization of NMY-2 relies on UNC-45 function. Surprisingly, although one would predict that NMY-2 does not attain its mature conformation in the absence of UNC-45, it is still able to interact with actin implying that UNC-45 is dispensable for this association. This data shows that UNC-45 is involved not only for the function of muscle myosin but also in non-muscle myosin-dependent processes in the early *C. elegans* embryo.

1.5 Fungal UCS proteins

UNC-45 shares a domain (the UCS domain) with several other proteins that have been implicated in myosin-dependent processes (Figure 1.11) (Hutagalung et al., 2002; Yu, 2003). The UCS domain is present in proteins of species ranging from fungi to mammals indicating that the role of this domain is of great importance to eukaryotes. Three such homologues of the fungal kingdom have been studied in some detail.

1.5.1 She4p in *Saccharomyces cerevisiae*

In *Saccharomyces cerevisiae* the UCS protein, She4p, is required for molecular transport of the *ASH1* mRNA to the daughter cell which is required to repress the expression of the *HO* endonuclease thereby repressing mating type switching (Bobola et al., 1996; Jansen et al., 1996). Additionally *she4* mutants also display endocytosis defects, failure to properly maintain actin polarization and altered growth at elevated temperature (Wendland et al., 1996). These defects arise due to the necessity of the interaction between the UCS domain of She4p and the motor domains of myosins (Jansen et al., 1996; Toi et al., 2003; Wesche et al., 2003). Co-immunoprecipitation studies indicate that She4p interacts with type I (Myo3p and Myo5p) and type V (Myo2p and Myo4p) myosins only (Wesche et al., 2003). These interactions are surprising if one compares She4p to other UCS proteins as UCS proteins predominantly interact with type II myosin molecules. However yeast two-hybrid results indicate an additional interaction with the only type II myosin found in yeast (Myo1p) (Toi et al., 2003; Wesche et al., 2003). She4p is required to localize Myo3p, Myo4p and Myo5p to the bud cortex while its own localization depends on the function of Myo2p, reminiscent of the dependency on

NMY-2 for UNC-45 localization (Kachur et al., 2004). The interaction between She4p and Myo5p is enhanced at higher temperatures consistent with She4p's potential chaperone activity. It is possible that the interactions between She4p and these myosin molecules are weak or transient, only becoming strong under conditions that do not favor maintenance of the properly folded state of the myosin motor domain.

1.5.2 CRO1 in *Podospora anserina*

CRO1 was discovered in the fungus, *Podospora anserina*, via a mutation that results in impaired sexual development. *P. anserina* undergoes a switch from a syncytial to a cellular cell fate. Following fertilization both mating types contribute nuclei that will divide in the cytoplasm but these nuclei do not fuse except in the dikaryotic upper cell. This cell is the product of hook shaped crozier cells, which when wildtype, forms three cells: the dikaryotic upper cell which will differentiate into an ascus capable of meiosis, and two uninucleate cells which are able to fuse later to form a new upper cell. The *cro1-1* mutants display croziers that can contain dozens of nuclei, instead of the normal two due to a lack of proper cellularization within the crozier. Meiosis can still occur in the *cro1-1* asci but they display abnormal spindle positioning and abnormal microtubule (tubulin) and microfilament (actin) organization. Wildtype actin belts delineate future septa sites. Though there is no direct link between CRO1 and myosin function it is hypothesized that the disorganized spindle in *cro1-1* mutants eliminates signals that designate the midline for future cellularization and therefore actin belts do not form there.

1.5.3 Rng3p in *Schizosaccharomyces pombe*

Likewise, proper function of Rng3p, another UCS protein, is necessary for the assembly of the actomyosin contractile ring in *Schizosaccharomyces pombe* (Wong et al., 2000). Rng-3-null cells are unable to undergo cytokinesis due to improper organization of the actomyosin ring. Rng3 interacts with the head domain of one of *S. pombe*'s two type II myosins, Myo2p and the Hsp90 homologue, Swo1p (Mishra et al., 2005). Though Rng3p function is not required for Myo2p accumulation at the division site, it is required for maintenance of the actomyosin ring. Rng3p and Swo1p localize to the division site in *myo2* mutants but this localization is less apparent in wildtype cells (Lord and Pollard, 2004; Mishra et al., 2005; Wong et al., 2000). Furthermore, the addition of Rng3p, specifically the UCS domain of Rng3p, to purified Myo2p increases the actin binding and motility activity of Myo2p (Lord and Pollard, 2004). Wong *et al.* (2000) hypothesized that Rng3p may transiently localize at the division site in wildtype cells but, when Myo2p is abnormal, Rng3p gets trapped at the division site due to failed actomyosin interaction or faulty myosin assembly consistent with its proposed role as a myosin chaperone. However Lord and Pollard suggest that the role of Rng3p is one of a myosin activator, not a chaperone due to its presence in the contractile ring. It may be possible that Rng3p is required for both the folding of myosin *and* the maintenance of its activity following its localization to the contractile ring.

1.6 Mammalian *unc-45*

Two UNC-45 homologues have been reported in mammals, termed UNC45A (previously known as GC UNC45 or SMAP1) and UNC45B (previously known as SM UNC45) (Price et al., 2002). The general cell (GC) homologue (*Unc45a*) is expressed in all mammalian tissue types examined, while the striated muscle (SM) homologue (*Unc45b*) is expressed only in tissue samples containing muscle, implying compartmentalization of the protein's function based on tissue type. Price et al (2002) examined expression of *Unc45a* and *Unc45b* in the C2C12 murine myoblast cell line and found only *Unc45a* to be expressed during the proliferation stage. Expression levels steadily decrease with maturation. *Unc45b*, on the other hand is first expressed during myoblast fusion, displaying highest expression levels in young myotubes when myofibrils are being assembled into functional contractile structures. Antisense experiments using the mouse C2C12 skeletal myogenic cell line demonstrate that UNC45A is required for cell proliferation and myocyte fusion while UNC45B acts later, in myocyte maturation and formation of striation. These data therefore imply UNC45A's role in development may parallel the role of UNC-45 in cytokinesis in the early *C. elegans* embryo while UNC45B functions in differentiating and mature muscle.

Additionally, UNC45A in conjunction with Hsp90 is involved in chaperoning the progesterone receptor (PR) (Chadli et al., 2006). UNC45A interacts with Hsp90 through its TPR domain but does not interact with Hsp90's common C-terminal TPR-interacting domain, located in the carboxyl terminus (Brinker et al., 2002; Scheufler et al., 2000). UNC45A interacts with the first 332 amino acids of Hsp90, within the ATP-binding domain. PR complexes that contain UNC45A display reduced hormone binding

capabilities implying UNC45A is likely involved in early/intermediate stages of PR maturation and that an unknown, later-acting protein may be involved in late maturation of the progesterone receptor.

1.7 Zebrafish (*Danio rerio*) as a model system

While *C. elegans* have some widely adopted benefits of use as a model system, such as transparency, ease of use and hermaphroditism that allows for ease in genetic studies, its simplicity demands that its development differs drastically from that of vertebrates. Ideally to study human disease and disorder the use of mammals would prevail. However mammals develop *in utero* which prevents observation of development and thereby developmental abnormalities. Moreover mammalian systems are costly to raise and maintain, produce small brood sizes and have a long generation time. Zebrafish is therefore an attractive intermediate system as it is a vertebrate so development resembles mammalian development more closely than the invertebrate species used in genetic studies and it does not suffer from many of the limitations of mammals.

Zebrafish are small (approximately 3.5 cm) fish originating in India. They are easy to maintain in a laboratory setting, reproduce easily and have high fecundity. They also have a short generation time (approximately 3 months) and external fertilization. Because of the transparency of the embryos and the external fertilization, all stages of development can be easily observed.

In terms of studying gene function in zebrafish, there are well-established gene knockdown techniques (antisense morpholinos) commonly used in zebrafish. Also large

scale genetic screens are possible as F1 haploid females can be screened for defects and can develop with relatively few problems. However the zebrafish genome underwent a partial or complete duplication since divergence from mammals 420 million years ago (Amores et al., 1998). This duplication can pose some issues related to gene redundancy (Taylor et al., 2003).

As mentioned, morpholinos have been used for the past seven years to phenocopy the effects of a mutation in a gene of interest in zebrafish (Nasevicius and Ekker, 2000). These morpholinos are stable oligonucleotides that are composed of a nucleic acid base linked to a morpholine-ring backbone and are easily delivered into the cytoplasm by microinjection of 1-8 cell embryos. They are designed to specifically bind near the translation start site or a splice site on the target RNA and therefore interfere with protein expression by sterically blocking translation or splicing. Unfortunately morpholinos targeted to more than 30 bases downstream of the translation start site tend to show decreased efficiency at reducing protein levels and are therefore avoided if possible.

Despite the identification of mutations resulting in myopathies in humans, the use of mammalian models is limited due to the potential early embryonic lethality that often results from defects in muscle development. This drawback does not exist in zebrafish due to its external fertilization and development. For example, the elimination of dystroglycan during embryogenesis causes muscular dystrophy in zebrafish whereas dystroglycan-null mice die pre-implantation (Parsons et al., 2002; Williamson et al., 1997). Similarly, zebrafish that have a mutation in the *atp2a1* gene are viable but display motility defects (Gleason et al., 2004) whereas mice lacking ATP2A1 function die at birth due to respiratory failure stemming from defects in the diaphragm muscle (Pan et

al., 2003). Because zebrafish embryos do not require circulation for the first several days following fertilization, heart development can be studied without the interference of any hypoxic effects. Mutations in the sarcomeric proteins titin and cardiac troponin T that cause cardiac dysfunction have been identified in zebrafish (Sehnert et al., 2002; Xu et al., 2002). Human mutations in either of these proteins are associated with potentially fatal familial cardiomyopathies (Gerull et al., 2002; Thierfelder et al., 1994). Therefore zebrafish represent an ideal system to study genes involved in muscle and cardiac function.

1.8 Myogenesis in zebrafish

The process of muscle development in vertebrates is one that is complex and has been long studied (Pownall et al., 2002). Myogenesis in zebrafish is regulated similarly to other vertebrates but there do exist some differences. Trunk muscle originates from structures called somites, chevron shaped blocks of cells whose segments bud off from anterior end of presomitic mesoderm. In addition to containing the muscle progenitor cells, somites also give rise to axial skeleton in zebrafish and the dermis in other organisms. Muscle precursors are located lateral to the notochord and express the myogenic regulatory factors, *myod* and *myf5*. Within this myotome are the precursors for slow muscle cells, muscle pioneer cells, fast muscle and medial fast fiber cells (see Table 1.1 for list of known striated muscle myosins in zebrafish). Adaxial cells are found directly adjacent to notochord are precursors for slow muscle. Most of these adaxial cells migrate laterally where they differentiate into slow muscle, just under the skin. Muscle

pioneers are a subset of slow muscle that express Engrailed and do not migrate. These cells may aid in guiding motor neurons. Medial fast fibers, whose role and origin is unknown, also express Engrailed but are known not to be muscle pioneer cells as they do not express slow muscle myosin. The remainder of the muscle progenitor cells are fated to become fast muscle and therefore express fast muscle myosin. (Figure 1.12A).

myod and *myf5* are two of the four known myogenic regulatory factors (MRFs). These genes are required for muscle determination and maintenance and show some functional redundancy (Rudnicki et al., 1993). The other two MRFs, *myogenin* and *mrf4* are expressed slightly later and are involved in differentiation, not determination.

The genetic regulation of myogenesis is complex and not completely understood at the moment (Ochi and Westerfield, 2007). For this reason, a simplified model will be presented here. The expression of *myod* relies heavily on the action of fibroblast growth factors, *fgf8* and *fgf24* though these are not the only proteins involved in ensuring correct control of *myod* expression (Draper et al., 2003). In adaxial cells, *myod* regulation is controlled by the interactions between Hedgehog (Hh) proteins, Patched (Ptc) and Smoothened (Smo). In the absence of Hh, Ptc interacts with Smo to negatively regulate Hh signaling. However when Hh is present, Hh binds Ptc, sequestering it from Smo, thereby relieving the signal inhibition. Hh is a secreted protein so in order to maintain a sharp gradient of Hh-responsive cells, Hhip negatively regulates Hh by binding it and preventing its diffusion to neighboring cells (Ochi et al., 2006). There are many regulators and downstream components of the Hedgehog pathway. The molecular mechanism by which Scube2, another secreted protein, acts is debated but it is known to be involved in the regulation of this pathway. Animals possessing mutations in *scube2*

display disrupted *myod* expression and fail to form muscle pioneer cells (Woods and Talbot, 2005). Similarly mutations in *gli2* also reduce *myod* expression, demonstrating that it too is involved in the Hh-dependent slow muscle myogenesis pathway (Figure 1.12B) (Karlstrom et al., 1996).

Despite all that is known about slow muscle determination in zebrafish, little is known about how fast muscle is specified. It has been shown that retinoic acid (RA) is involved in fast muscle development as *Raldh2*, a RA synthesizing enzyme, is expressed in the somites and it activates *fgf8* expression thereby promoting *myod* expression and fast muscle differentiation (Figure 1.12C) (Hamade et al., 2006). Additional studies will be required to further elucidate this pathway.

1.9 Zebrafish heart development

The zebrafish heart is a simple two-chambered organ, composed of a myocardium and an underlying endocardium but despite its simplicity, its development is very complicated (Stainier, 2001; Yelon, 2001). It starts as a tube that is then separated into two distinct chambers, the atrium and the ventricle, each expressing its own myosin heavy chain (see Table 1.1). Expression of cardiac determination factors begins in the anterior lateral plate mesoderm (ALPM), lateral to the embryonic midline. *Nkx2.5*, a transcription factor considered to be one of the early cardiac determination markers, is expressed in a subset of *gata4* expressing cells in the ALPM. Most of these cells become cardiomyocytes. During differentiation, these cells begin to express genes encoding components of the sarcomere such as *myl7* (a cardiac myosin light chain), *myh6* (the

atrial myosin heavy chain) and *vmhc* (the ventricular myosin heavy chain). *myh6* and *vmhc* are each expressed in a subset of *myl7*-expressing cells, indicating the regions of the ALPM that will become each of the two chambers (Figure 1.13A). How these regions are determined prior to *myh6* and *vmhc* expression is unknown, however retinoic acid is thought to play a role as the addition of exogenous retinoic acid appears to inhibit ventricular development (Stainier and Fishman, 1992).

Following determination, the cardiomyocyte precursors migrate toward the midline where the two populations of cells meet and fuse as two sheets. This creates a cardiac cone that, through complex cell movements and rearrangements, becomes a linear tube. Cardiac morphogenesis continues by transforming this single tube into a functional looped, two-chambered heart capable to circulating blood. Zebrafish hearts loop so that the ventricle is anterior to and to the right of the atrium. The process by which this occurs is thought to rely on the physical forces created by circulation (Hove et al., 2003). A mature zebrafish heart also requires a valve located between the atrium and the ventricle to prevent backflow of blood. This valve originates as a cardiac cushion, formed by a specialized population of endocardial cells. Like myogenesis, cardiogenesis is an extremely complicated process that requires further research if we are to ever fully understand how the heart develops.

1.10 Other known myosins in zebrafish

In addition to the conventional type II myosins discussed above (Table 1.1) numerous unconventional myosins (of types I, III, V, VI, VII, IX, X and XI) have been

identified in zebrafish, though few of these have been studied (Table 1.2). Mutations have been identified and further characterized in three of these myosin-encoding genes, *myh11*, *myo6b* and *myo7a*.

Type VI myosin is known to walk along actin in the opposite direction from most myosins and has been shown to have a role in vesicle movement and formation (Hasson, 2003; Homma et al., 2001; Nishikawa et al., 2002; Wells et al., 1999). Mutation in type VI myosin has been identified as the cause of nonsyndromic hearing loss in humans (Ahmed et al., 2003; Melchionda et al., 2001). In contrast to humans, zebrafish have two *myo6* homologues with *myo6a* being expressed ubiquitously early with expression becoming predominant in the brain, gut and kidney at later stages. *myo6b* is expressed mainly in the hair cells of the ear and lateral line, the organs responsible for sensing sound, gravity and water movement. It was found that the *satellite* line of zebrafish have mutations in the *myo6b* locus (Kappler et al., 2004; Seiler et al., 2004). These fish swim in an uncoordinated fashion consistent with balance defects due to malformation of the hair cells (Granato et al., 1996; Nicolson et al., 1998). Another case of myosin function being required for balance is seen in the strain *mariner*. This strain also displays defects in hair cell function though the causative mutation in this strain is in the *myo7a* gene which has been implicated in Usher 1B syndrome and other forms of inherited human deafness (Ernest et al., 2000; Liu et al., 1997a; Liu et al., 1997b; Weil et al., 1995; Weil et al., 1997). Similar to *myo6b*, *myo7a* is expressed in the developing inner ear and the lateral line and the *mariner* mutations also result in alterations in the hair cell organization. In this case the bundles of hair cells are frequently splayed instead of being

tightly bundled. This demonstrates that at least two different classes of myosin are required for proper sensory organ development in zebrafish.

The last currently known zebrafish strain with a mutation in a non-type II myosin is *meltdown* (*mlt*). These fish display cystic expansion of the posterior intestine due to a gain of function mutation in the smooth muscle myosin, *myh11* (Wallace et al., 2005). This mutation results in constitutive ATPase activity and no motor function. This disrupts the smooth muscle cells and allows for invasion of epithelial cells resulting in intestinal cysts. These fish show ectopic activation of genes that are known to play roles in cancer cell invasion and partial knockdown of these genes are able to partially rescue the *mlt* phenotype. These studies reveal the strength of zebrafish as a model for studying the function of myosin and the processes in which it is involved.

1.11 Specific goals of this thesis

Although Price *et al.* (2002) showed UNC45B to be involved in the maturation of C2C12 myocytes, the vertebrate role outside of cell culture has not been examined. To test whether UNC-45 is essential for the development and normal function of vertebrate skeletal and cardiac muscle in a live animal, we examined the *unc45b* homologue using the zebrafish, *Danio rerio* as a model. The goals of this thesis were to:

1. identify vertebrate homologues of *unc-45*
2. examine expression pattern of vertebrate *unc45* using northern analysis, *in situ* hybridization and RT-PCR
3. determine the localization of Unc45b in zebrafish

4. examine the zebrafish *unc45b* knockdown phenotype during development
5. examine the phenotype of mutants that, by preliminary characterization appeared to be similar to *unc45b* morphants

1.13 Summary of this thesis

I have identified the *unc45a* homologue present in mammals and zebrafish as well as the zebrafish *unc45b* homologue. I show that *unc45a* is widely expressed in vertebrates while *unc45b* is expressed only in tissues that contain striated muscle. In zebrafish embryos, reduction of Unc45b protein levels achieved through antisense morpholino injections results in paralysis and cardiac dysfunction. The motility defects, and presumably the cardiac defects, observed are due to sarcomeric disorganization and loss of myosin filaments. Embryos lacking Unc45b also display abnormal craniofacial features. I also identified and characterized three mutant strains of zebrafish that display *unc-45b* morphant-like phenotypes. Two of these strains, *herzschlag* and *still heart*, suffer from skeletal muscle disorganization and cardiac abnormalities while *jam* only displays minor motility defects with unknown underlying cause. Neither the *herzschlag* nor *still heart* phenotypes are caused by mutations in *unc45b* despite the similarities between phenotypes. This research reports the importance of UNC-45 in the function of vertebrate skeletal and cardiac muscle, likely by aiding in the folding or assembly of myosins specific to these tissue types.

Table 1.1 Known type II myosins in zebrafish striated muscle

Symbol	Name	Expression pattern	Known mutant?	References
<i>myh6</i>	myosin, heavy polypeptide 6, cardiac muscle, alpha	cardiac (atrial) mesoderm	<i>weak atrium</i>	Berdougo et al., 2003
<i>myhc4</i>	myosin heavy chain 4	adaxial cells, fast muscle cells, muscle pioneer, slow muscle cells	None	Bryson-Richardson et al., 2005
<i>myhz1</i>	myosin, heavy polypeptide 1, skeletal muscle	somites	None	Xu et al., 2000
<i>myhz2</i>	myosin, heavy polypeptide 2, fast muscle specific	somites, pectoral fin musculature, cranial muscle, musculature	None	Thisse et al., 2001 Peng et al., 2002
<i>smyhc1</i>	slow myosin heavy chain 1	adaxial cells, somites, slow muscle cells, heart	None	Rauch et al., 2003; Bryson-Richardson et al., 2005
<i>smyhc11</i>	slow myosin heavy chain 2, like	adaxial cells, somites, slow muscle cells, mandibular muscle	None	None
<i>vmhc</i>	ventricular myosin heavy chain	cardiac (ventricular) mesoderm, musculature	None	Yelon et al., 1999

Table 1.2 Other known myosins in zebrafish

Symbol	Name	Expression pattern	Known mutant?	References
<i>myh9</i>	myosin, heavy polypeptide 9, non-muscle	nd	None	
<i>myh10</i>	myosin, heavy polypeptide 10, non-muscle	nd	None	
<i>myh11</i>	myosin, heavy polypeptide 11, smooth muscle	gut	<i>meltdown</i>	Wallace et al., 2005
<i>myo1e</i>	myosin 1e	nd	None	
<i>myo3a</i>	myosin IIIa	axis, polster, notochord	None	Thisse and Thisse, 2005
<i>myo5a</i>	myosin Va	epidermis, olfactory placode, otic vesicle, lateral line, pronephric duct, pigment cells	None	
<i>myo5b</i>	myosin Vb		None	Thisse et al., 2001
<i>myo6a</i>	myosin VIa	brain, gut, kidney	None	Kappler et al., 2004; Seiler et al., 2004
<i>myo6b</i>	myosin VIa	sensory epithelium of ear, lateral line	<i>satellite</i>	Kappler et al., 2004; Seiler et al., 2004
<i>myo7a</i>	myosin VIIa	otic vesicle, lateral line	<i>mariner</i>	www.zfin.org
<i>myo9b</i>	myosin IXb	nd	None	

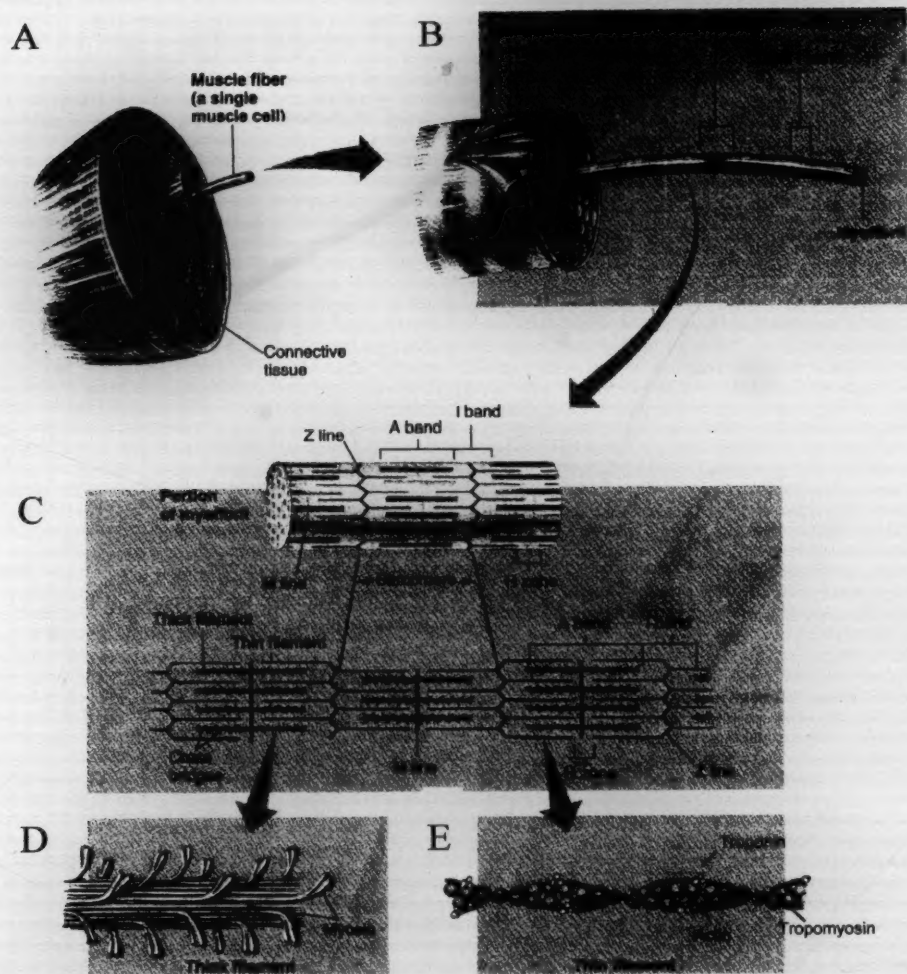


Figure 1.1 Striated muscle structure and organization

(A) Magnified representation striated muscle with focus on a single muscle cell. (B) Representation of a single muscle fiber (muscle cell) with focus on a single myofibril. (C) Portion of a myofibril demonstrating organization of thin and thick filaments within a sarcomere. (D) Enlargement of a thick filament composed of myosin. (E) Enlargement of a thin filament composed mainly of actin, associated with the tropomyosin-troponin complex. Adapted from Sherwood (2001).

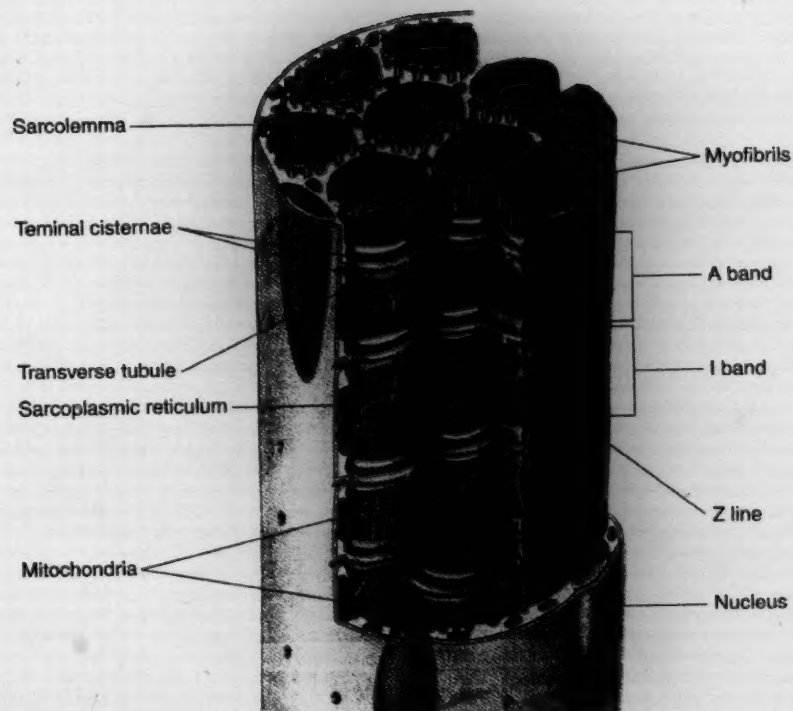


Figure 1.2 Transverse tubules and sarcoplasmic reticulum

Representation of a muscle cell in relation to the transverse tubules (T-tubules) and the sarcoplasmic reticulum. T-tubules transduce the action potential created by stimulation by neurons to the sarcoplasmic reticulum which then releases calcium into the cytoplasm, resulting in muscle contraction. Adapted from Fox (2004).

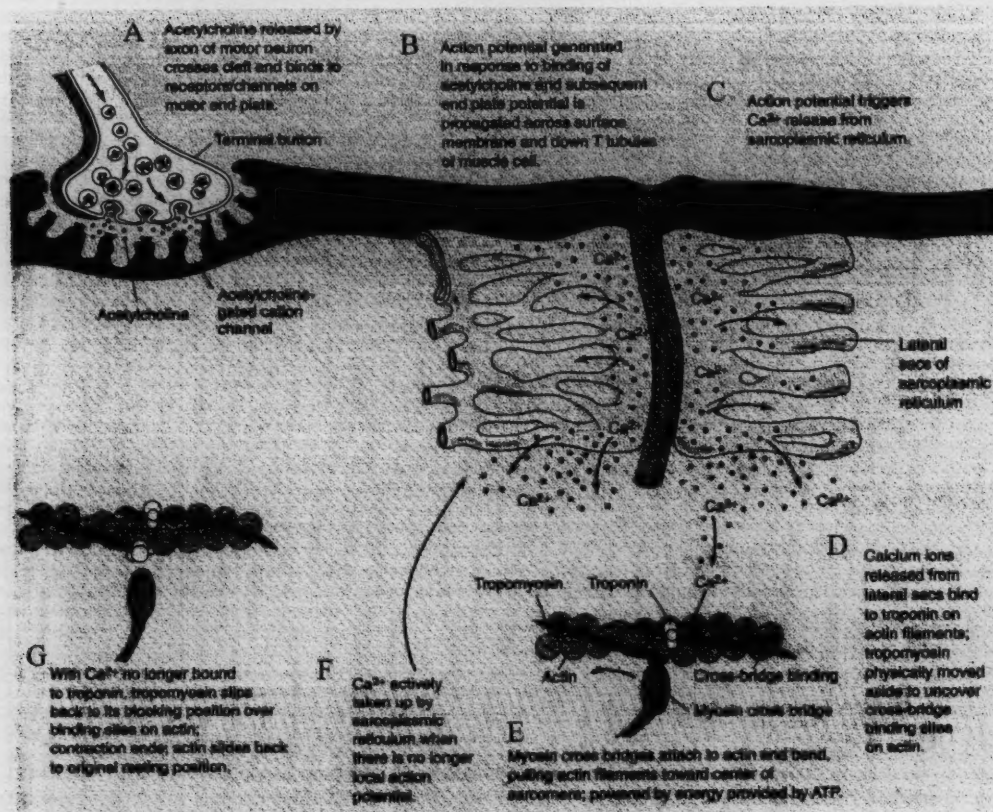


Figure 1.3 Mechanism of muscle contraction

Muscle contraction occurs through a standard pathway. (A) The neurotransmitter acetylcholine is released by the motor neuron. (B) Action potential is transduced through T tubules into muscle cell (C) Calcium is released from sarcoplasmic reticulum into cytoplasm. (D) Calcium binds troponin which results in movement of tropomyosin to uncover myosin-binding sites on actin. (E) Myosin and actin interact, myosin bends creating power stroke which physically pulls actin filaments towards the center of the sarcomere. (F) In absence of stimulation, calcium is taken up into sarcoplasmic reticulum. (G) Without calcium, tropomyosin moves to block actin's myosin-binding sites. Adapted from Sherwood (2001).

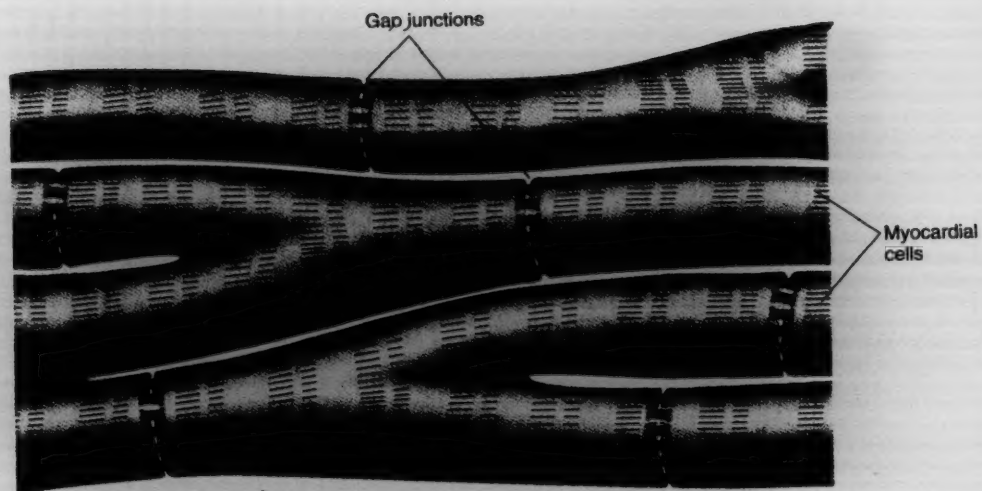


Figure 1.4 Cardiac muscle organization

Representation of cardiac muscle showing the connections between adjacent myocardial cells through gap junctions located at the ends of the cells. Gap junctions allow for signals to be transduced through all cardiac cells simultaneously by connecting the cytoplasm of adjacent cells. Adapted from Fox (2004).

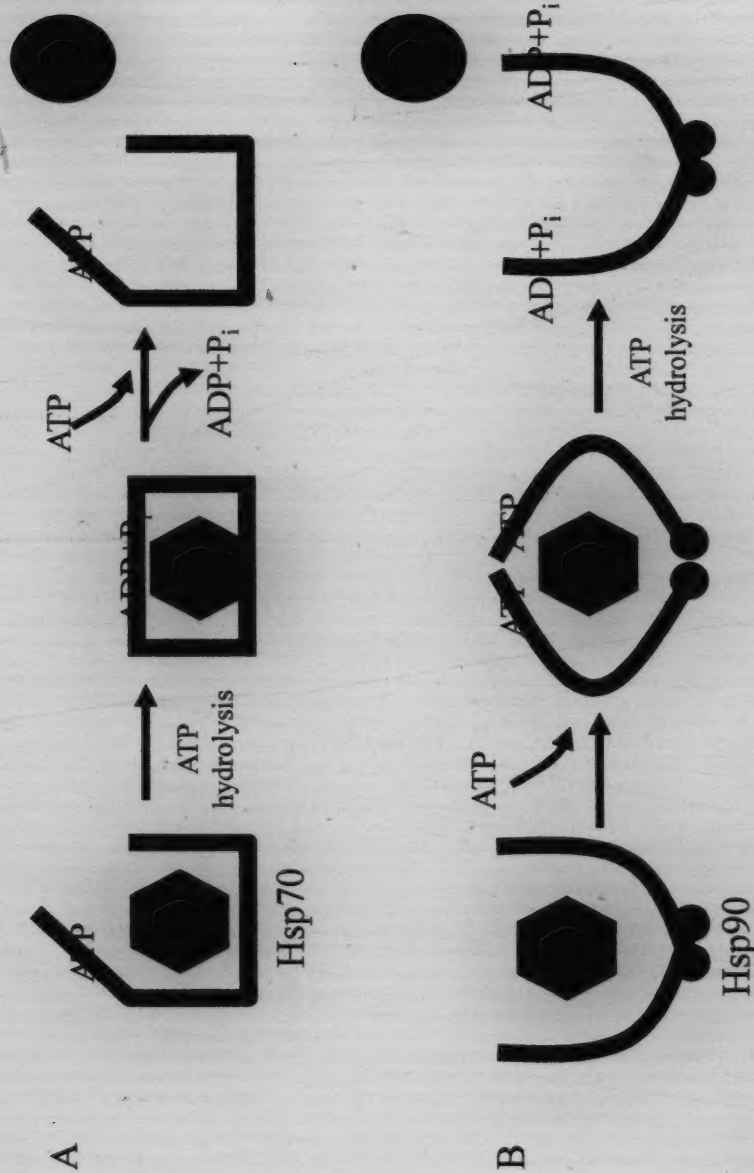


Figure 1.5 Mode of action of Hsp70 and Hsp90

(A) The substrate protein interacts with Hsp70 when it is bound by ATP at its N-terminus. When ATP is hydrolyzed, the Hsp70 "clamp" closes, trapping the substrate protein until the ADP and P_i molecules are released and a new ATP molecule binds. (B) Hsp90 forms a dimer at its C-terminus and acts differently than Hsp70. The substrate protein binds to the Hsp90 dimer when in its nucleotide-free state. When ATP binds, the Hsp90 dimer changes its conformation trapping the substrate protein. The substrate protein remains bound until ATP hydrolysis occurs.

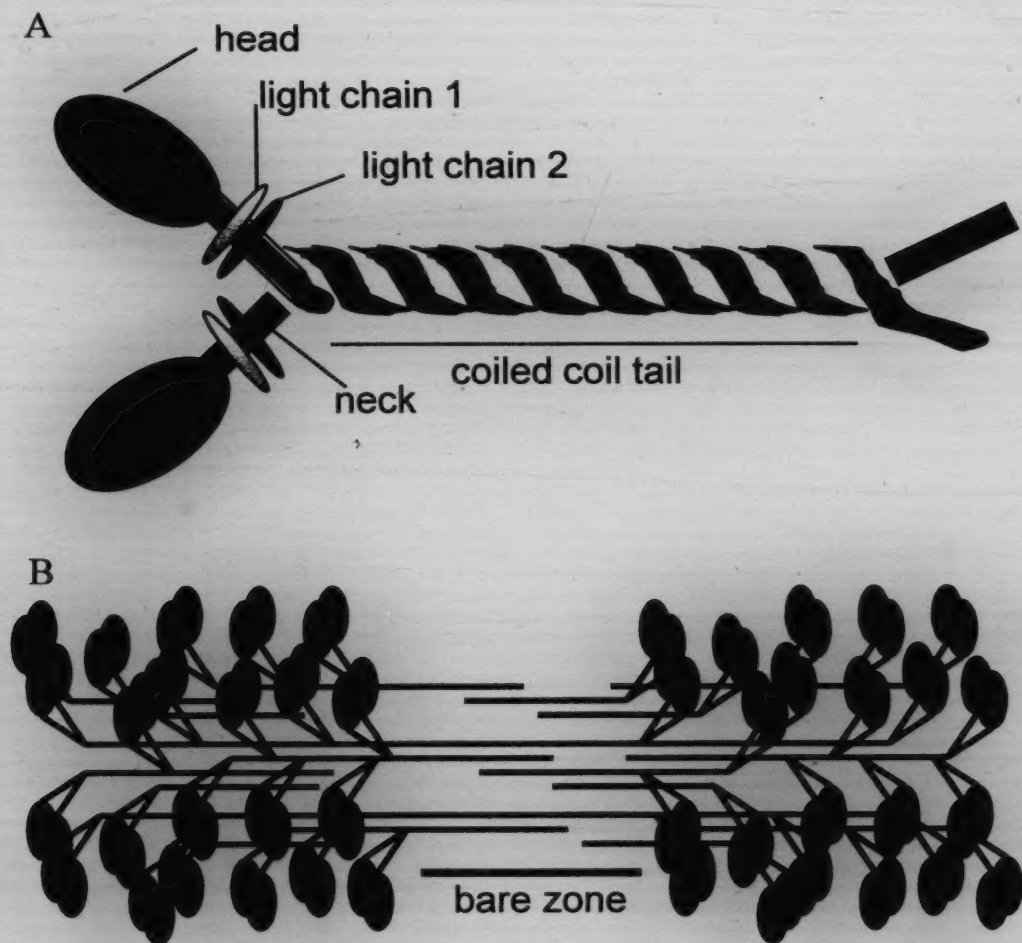


Figure 1.6 Myosin and thick filaments

(A) Representation of a single myosin molecule. Myosin heavy chains form dimers through association of their tail domains. Each heavy chain interacts with two light chains at their neck domains. (B) Myosin dimers interact with each other to form thick filaments. In the center of this filament, molecules are arranged in an antiparallel fashion with the tails pointed towards the center, creating a head-free bare zone. Myosin molecules pack in parallel with each other distal to this bare zone to create the thick filament.



Figure 1.7 Protein domains of *C. elegans* UNC-45
 UNC-45 is composed to three major domains. The N-terminal consists of the tetratricopeptide repeat (TPR) domain (pink), the middle third makes up a unique central domain (purple) and the C-terminus contains the UCS (UNC-45/CRO-1/She4p) domain (blue). 4-point stars indicate positions of lethal, nonsense alleles. 5-point stars indicate positions of temperature-sensitive, missense alleles. Numbers above stars represent the position of the amino acid affected in each allele.

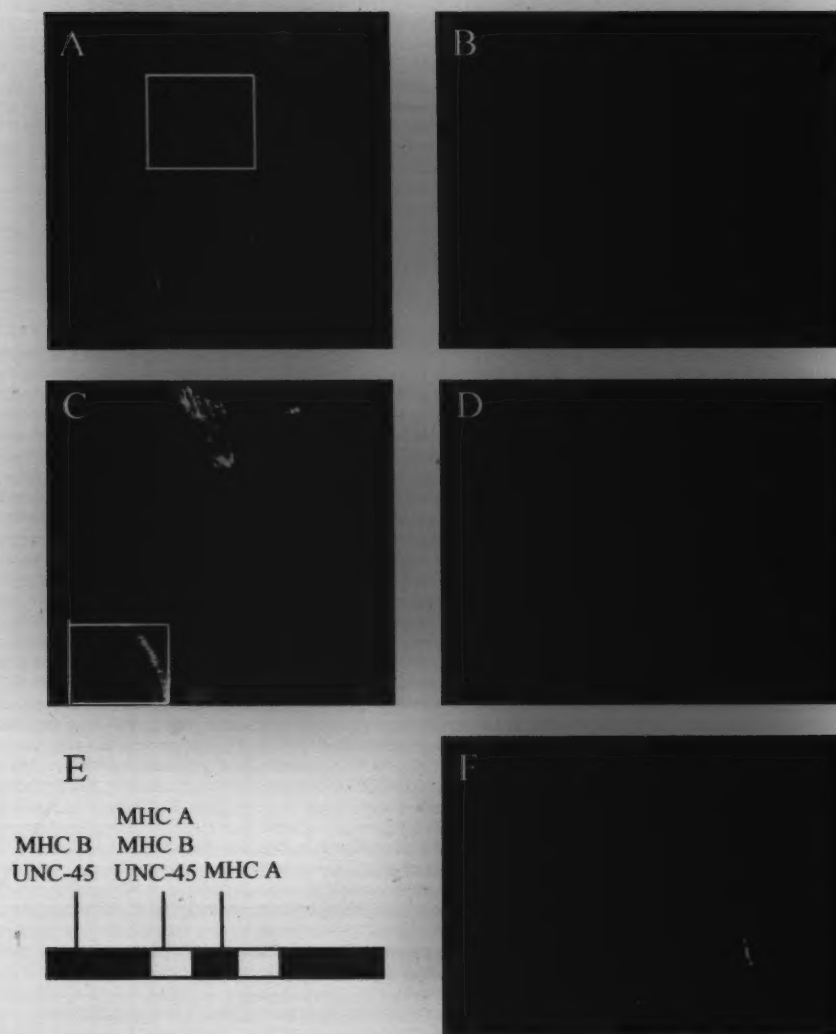


Figure 1.8 Localization of UNC-45 in *C. elegans*

(A-D) Adult *C. elegans* body wall muscle stained with antibodies against UNC-45 (green) and (A,B) MHC A or (C,D) MHC B (red) demonstrating co-localization of UNC-45 with MHC B but not MHC A. B and D are magnifications of the boxed regions of A and C, respectively. Anterior-posterior axis runs vertically. (E) Diagram of the *C. elegans* thick filament indicating localization of MHC A, MHC B and UNC-45. (F) Approximately 20-cell embryo stained with antibodies against UNC-45 (green) and NMY-2 (red), demonstrating co-localization of UNC-45 and NMY-2. Yellow represents regions of overlap in staining patterns. Anterior-posterior axis runs horizontally. Adapted from Horlick (2005) and Kachur *et al.* (2004)

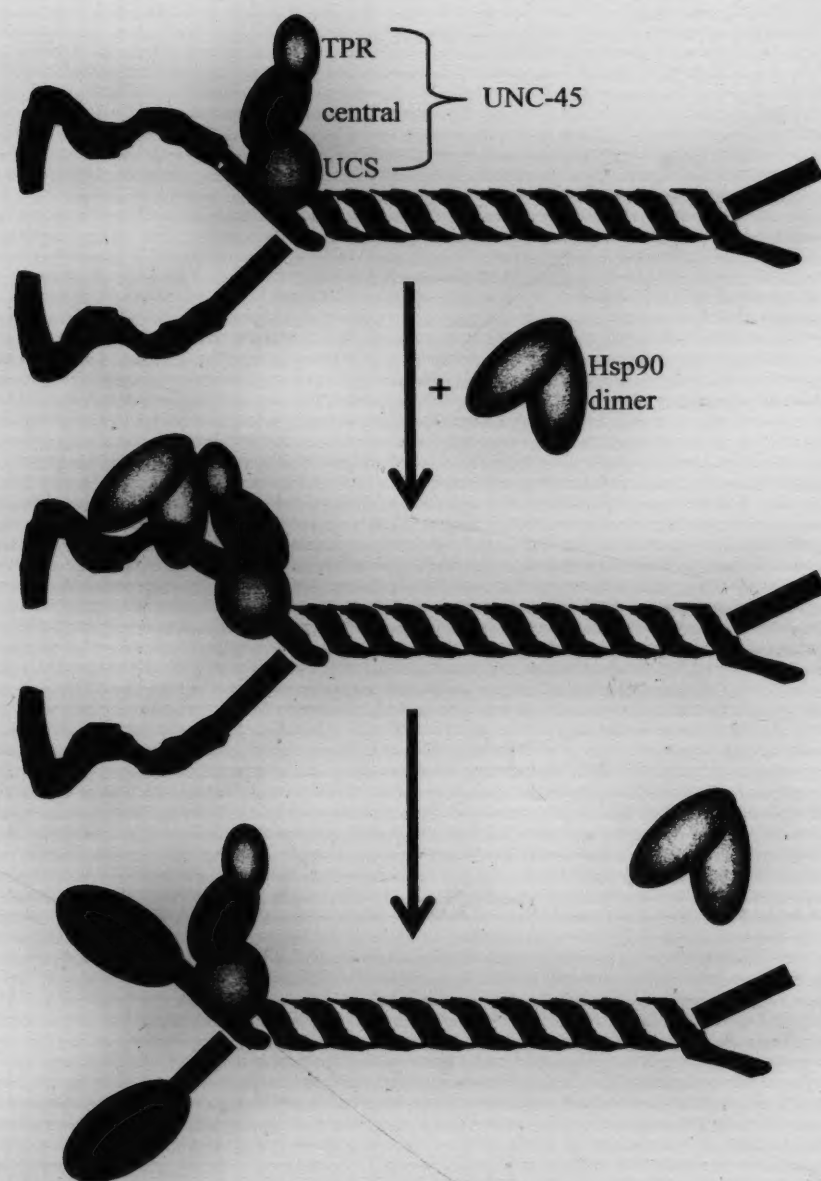


Figure 1.9 Model of UNC-45 as a myosin co-chaperone
 UNC-45 is associated with the neck domain of myosin through its UCS domain. To aid in folding, the Hsp90 dimer interacts with the TPR domain of UNC-45 and the head domain of myosin. Once myosin nears its mature conformation, Hsp90 is released but UNC-45 remains associated with the myosin molecule.

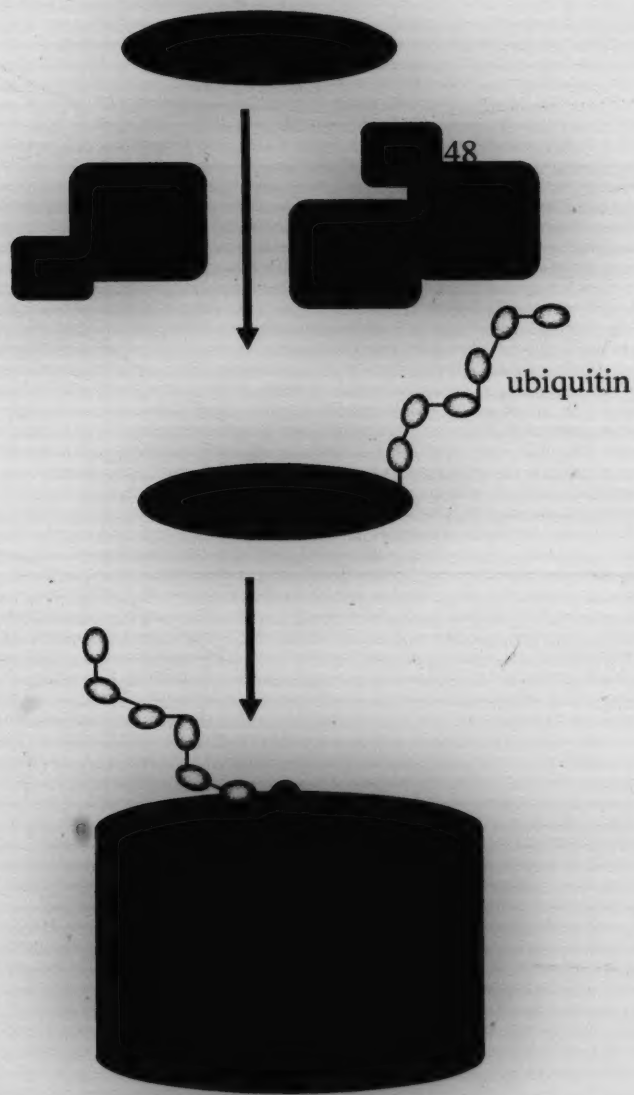


Figure 1.10 Model of regulation of UNC-45 levels

UNC-45 levels are regulated through ubiquitylation by a E3/E4 complex composed of CHN-1 (E3), UFD-2 (E4) and the ubiquitin-selective chaperone CDC-48. Following ubiquitylation, UNC-45 is targeted to the 26S proteasome for degradation. When proper balance of UNC-45 levels are not maintained sarcomeric defects are observed. Adapted from Hoppe (2004).

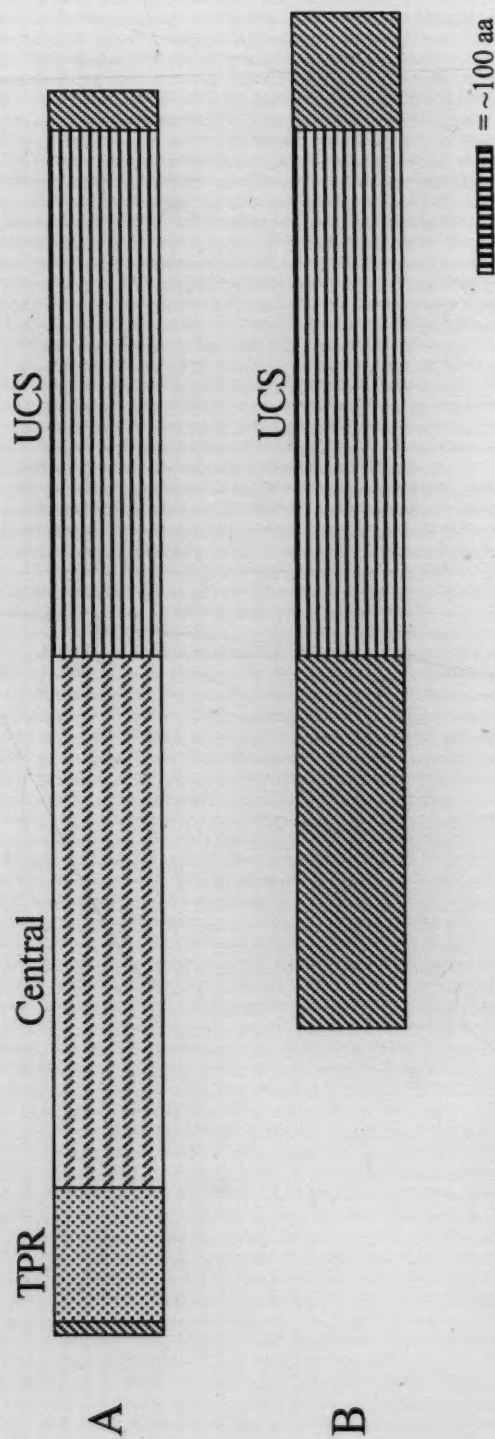


Figure 1.11 Structure of the UCS proteins.

Structure of the (A) metazoan and (B) fungal UCS proteins showing the TPR domain (dotted), the unique central domain (cross-hatched) and the UCS domain. (horizontal lines) Only the UCS domain is shared across metazoans and fungi.

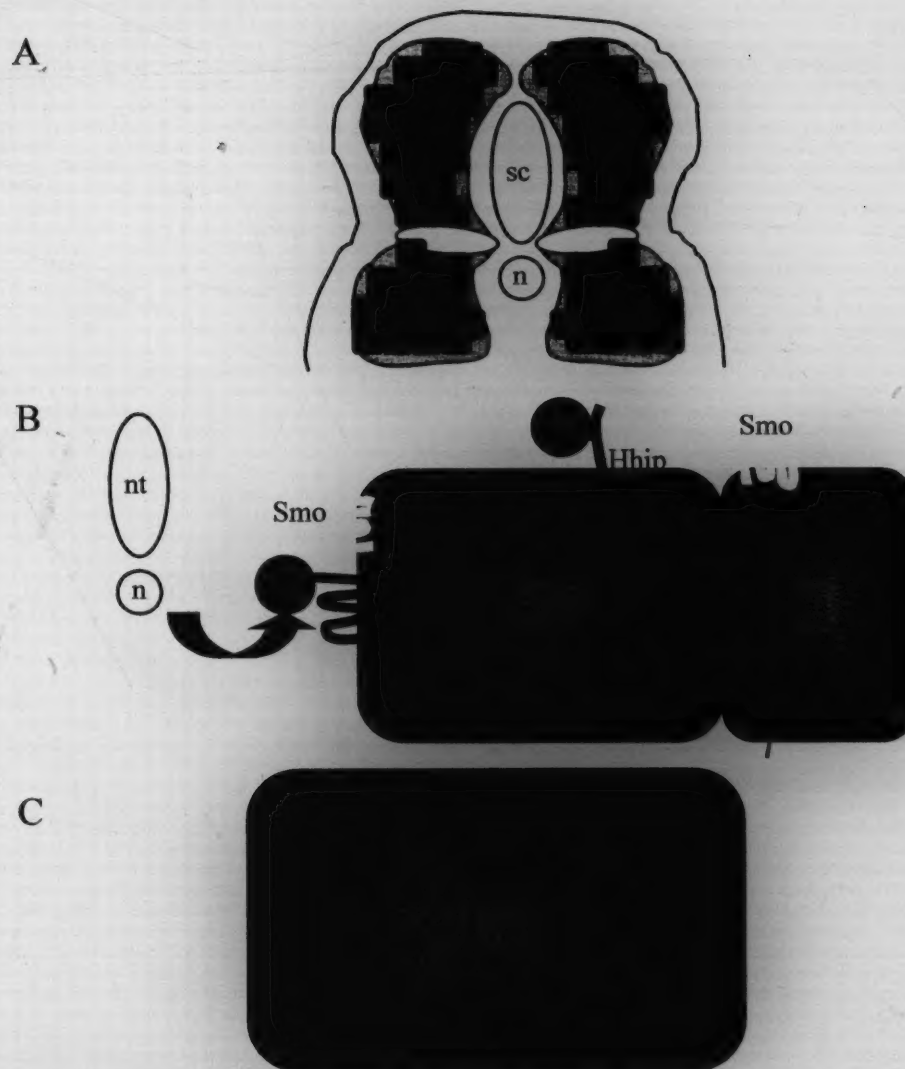


Figure 1.12 Myotome organization and muscle specification

(A) Placement of different muscle cell types in the zebrafish myotome at approximately 24 hpf. Slow muscle cells (red) have migrated laterally, leaving the Engrailed-expressing slow muscle cells (yellow) in their original position. Medial fast muscle cells (purple) are found deep within the muscle. The rest of the muscle is composed to fast muscle cells, (blue). (B) Determination of slow muscle cells is mediated by Hedgehog (Hh) signaling through Smoothened (Smo) and Gli, resulting in *myod* expression. (C) *myod* expression in fast muscle is mediated through retinoic acid and Fgf8. n, notochord; nt, neural tube; RA, retinoic acid; sc, spinal cord. Adapted from Ochi and Westerfield (2007)

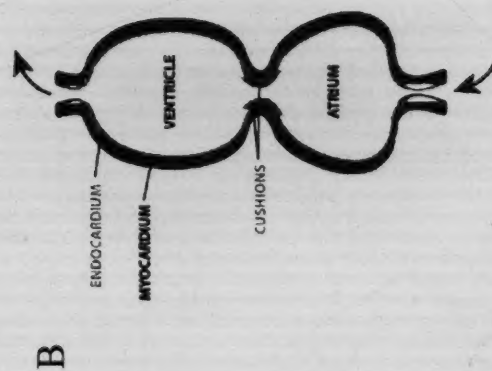
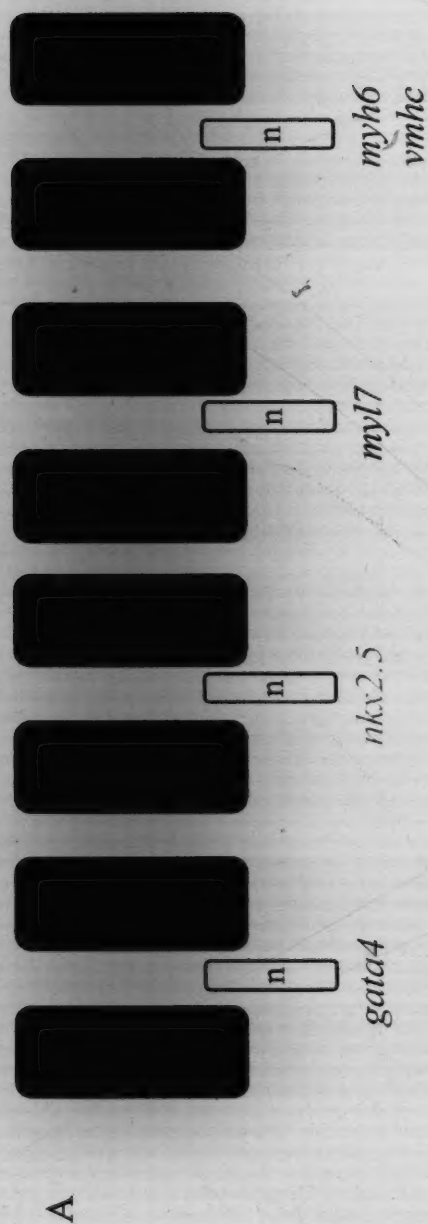


Figure 1.13 Cardiac specification and structure in zebrafish

(A) Gene expression patterns in anterior lateral plate mesoderm. *nkx2.5* is expressed in a subset of *gata4* expressing cells. *myl7* is expressed in differentiating cardiomyocytes. *myh6* and *vmhc* are expressed in subpopulations fated to become the two separate zebrafish chambers. (B) Representation of the two-chambered zebrafish heart prior to cardiac looping. The ventricle is located anterior to the atrium. The two chambers are separated by a cardiac cushion (which will eventually become an atrioventricular valve) formed from the endocardium. n, notochord. Adapted from Yelon (2001).

2. Materials and Methods

Primer sequences are provided in Appendix I.

2.1 Zebrafish strains used

AB

This inbred strain is considered wildtype. It was developed by the Streisinger lab in Eugene, OR and was obtained from the Zebrafish International Resource Center (Eugene, OR). This stock will be referred to as AB or wildtype throughout the text.

nacre (*nac*^{w2})

This strain carries a mutation induced by ENU in the *microphthalmia-associated transcription factor a* (*mitfa*) gene and lacks melanophores throughout development (Lister et al., 1999). This results in lack of pigmentation. This strain was provided by the Zebrafish International Resource Center (Eugene, OR).

herzschlag (*helf*^{g287}), *jam* (*jam*^{tr254a}), *still heart* (*sth*^{tm123a})

These strains carry mutations induced by ENU in currently unknown genes in the inbred Tu (Tuebingen) background. They were developed by the Nüsslein-Volhard lab in Tübingen, Germany (Chen et al., 1996). These strains were provided by the Max-Planck-Institut für Entwicklungsbiologie (Tübingen, Germany).

WIK

This strain was developed for use in genetic mapping. It was developed by the lab of Pascal Haffter.

2.2 Zebrafish care

Adult zebrafish were maintained according to standard procedures (Westerfield, 2000) and were kept at 28.5°C on a 14 hour light/10 hour dark cycle. Adults were housed in an aquatic facility supported and maintained by the lab of Andrew Waskiewicz. Adults were naturally spawned to obtain embryos. Embryos were raised at 28.5°C in embryo media. 0.003% 1-phenyl-2-thiourea (PTU) was added to reduce pigmentation where described in the text.

2.3 Bioinformatic identification of vertebrate *unc-45* homologues

To identify mammalian *unc45* homologues, BLAST searches were performed with the sequence of the *C. elegans* UNC-45 protein against the translated database (tblastn) using the NCBI BLAST server at www.ncbi.nlm.nih.gov/blast/. Two ESTs, one from humans and one from mouse, with sequence similarity to *unc-45* were identified (GenBank accession no. AL572718, BC004717, respectively) and the clones were obtained from Research Genetics (Invitrogen). The inserts were sequenced demonstrating that these clones each contained 3.2 kbp inserts corresponding to a full length human *UNC45* cDNA and a full length mouse *Unc45* cDNA. Following the publication of Price *et al.* (2002) it was determined that these clones represent mammalian *UNC45A* (formerly referred to as *gc unc-45*).

To identify a zebrafish *unc45* homologue, BLAST searches were performed as described above and two ESTs (GenBank accession nos. AI723219, AI883844) corresponding to a putative zebrafish homologue were identified. These clones were

obtained from Research Genetics (Invitrogen) were sequenced. Both clones contain a 3.8 kbp cDNA corresponding to the full *unc45* mRNA which encodes a 934 amino acid protein with 71% identity to mammalian UNC45B (Price et al., 2002). The identification of this gene has been independently published under the name *unc45r* (GenBank accession nos. AI883844, AI723219) (Etheridge et al., 2002) however, to be consistent with *Danio rerio* nomenclature, we have called this homologue *unc45b*.

The zebrafish homologue of *unc45a* was identified by performing BLAST searches against the zebrafish genomic and EST databases using the sequence of the *Fugu rubripes* Unc45a (Price et al., 2002). While no ESTs were found, genomic sequence corresponding to a zebrafish *unc45a* open reading frame was identified (Genbank accession no. AL935028.14) and primers flanking this region were designed (for cloning of zebrafish *unc45a* see section 2.6.1). Recently *unc45a* ESTs have been added to the database (Genbank accession nos. CO931586.1, DV598140.1) that were not available when I cloned *unc45a*.

2.4 Human Northern Analysis

A human *UNC45A* probe was made by gel purifying a fragment corresponding to the entire human *unc45a* cDNA using the Sephaglas BandPrep Kit (Amerhsham). Approximately 200 ng of template was labeled with ^{32}P dCTP using the T7 Quickprime Kit as described in the manufacturer's instructions. To prepare the control probe, 100 ng of human β *actin* cDNA (supplied by manufacturer) was labeled with ^{32}P dCTP as described above.

A human fetal multiple tissue northern blot (Clontech, Cat. No. 7780-1) and a human adult multiple tissue northern blot (Clontech Cat. No. 636815) prepared from poly A⁺ RNA were prehybridized for 1.5 hours in Hybrisol II (Chemicon International) at 65°C and then hybridized with the human *UNC45A* or the β *actin* radiolabelled probe overnight at 65°C. The following washes were performed at 65°C: 2x SSC, 15 min; 2x SSC, 15 min; 2x SSC, 0.1% SDS, 30 min; 0.1x SSC, 0.1% SDS, 10 min. Autoradiograph film was then exposed to the hybridized blots for seven days and developed.

2.5 Cloning of zebrafish *unc45a*

Following identification of genomic sequence corresponding to a zebrafish *unc45a* homologue (see section 2.3) primers were designed to amplify the coding region. PCR was performed using 0.5 μ L of cDNA prepared from 100 hours post fertilization (hpf) embryos by Jessica Smith using the primers ZF UNC45B FOR and ZF UNC45B REV (please note that these primers were designed and named predating the naming of this *unc45* homologue *unc45a*). The reaction conditions were: 94°C, 30 sec (94°C, 15 sec; 71°C, -1°C /cycle, 20 sec; 68°C, 5 min) for 10 cycles, (94°C, 15 sec; 61°C, 20 sec; 68°C, 5 min + 6 sec/cycle) for 25 cycles. PCR was performed in a MJ Research PTC-100 thermal cycler. The 2.7 kbp PCR product was purified using the Sephaglas BandPrep Kit (Amersham) and cloned into the pGEM-T vector (Promega).

To obtain the 3' UTR of *unc45a*, 3' RACE was performed. Nested PCR was performed using the ZF GCUNC45 3'RACE 2 primer and the Gibco adapter primer with

0.5 μ L of cDNA prepared from 100 hpf embryos as a template. The PCR reaction conditions were: 94°C, 1 min; 61°C, 30 sec; 72°C, 1 min, 30 sec, (94°C, 30 sec; 61°C, 30 sec; 72°C, 1 min, 30 sec) for 29 cycles. Nested PCR was then performed using the ZF GCUNC45 3'RACE 3 primer and an adapter primer (Gibco) using 0.5 μ L of the initial PCR as template. The PCR conditions were: 94°C, 1 min; 61°C, 30 sec; 72°C, 4 min, (94°C, 30 sec; 61°C, 30 sec; 72°C, 1 min, 30 sec) for 29 cycles. A resulting 1.5 kbp PCR product was purified using the Sephaglas BandPrep Kit (Amersham) and cloned into the pGEM-T vector (Promega).

The 3' RACE fragment was then subcloned into the *unc45a*:pGEM-T construct by digesting both constructs with *Nhe*I (a unique restriction enzyme site present within both PCR fragments) and *Nde*I (a restriction enzyme site present in the pGEM-T vector downstream of the cloned fragments). This was followed by purification of the desired fragments and ligation of the *Nhe*I/*Nde*I 3' RACE fragment with the *Nhe*I/*Nde*I *unc45a*:pGEM-T fragment.

2.6 Whole mount *in situ* hybridization

2.6.1 Preparation of DIG-labeled RNA probes

Plasmids to be used as templates in the preparation of the *in situ* probes were linearized with enzymes that cleave towards the 5' end of the insert, opposite to the promoter to be used to synthesize the probe. Following linearization the templates were gel purified using the Sephaglas BandPrep Kit (Amersham). Digoxigenin-labelled

antisense RNA probes were synthesized by *in vitro* transcription using SP6, T3 or T7 RNA polymerase (Roche Diagnostics) or the SP6, T3 and T7 Megascript kits (Ambion) and digoxigenin-11-UTP RNA labeling mix (Roche Diagnostics) according to the manufacturer's manual using approximately 1 µg of linearized template. Transcription was allowed to continue for 1-2 h at 37°C. The reaction was then treated with DNase for 15 min at 37°C. Lithium chloride was used to precipitate the RNA which was then resuspended in 20 µL of nuclease-free water and stored at -20°C.

2.6.2 *In situ* hybridization of mouse embryos

11.5 dpc and 14 dpc embryos were collected and fixed in 4% paraformaldehyde in PBS overnight at 4°C, washed in PBS and dehydrated through a series consisting of 30% ethanol, 50% ethanol and 70% ethanol in DEPC water and stored at 4°C.

The embryos were then rehydrated to PBS + 0.1% Tween-20 (PBSTw) with washes of 50% ethanol in PBSTw and 25% ethanol in PBSTw followed by 2 washes of PBSTw. The embryos were bleached for approximately 1.5 hours in 6% hydrogen peroxide in PBSTw and then washed with PBSTw. They were then permeabilized in 20 µg/µL proteinase K in PBSTw for (20 min, 11.5 dpc embryos) or (30 min, 14 dpc embryos), washed in 2 mg/mL glycine in PBSTw for 10 min, then washed in PBSTw. The embryos were then re-fixed in 4% paraformaldehyde, 0.2% glutaraldehyde for 20 min and washed in PBSTw.

Prehybridization was performed at 68°C in 50% formamide, 5x SSC, 1% SDS, 50 µg/mL yeast RNA and 50 µg/mL heparin in DEPC water for 2 h. Hybridization was

performed using 5 μ L of probe per 1.5 mL of prehybridization solution at 68°C overnight. Following hybridization embryos were washed at 68°C in 50% formamide, 5x SSC, 1% SDS for 30 min (3 times), then in 50% formamide, 2x SSC for 30 min (3 times), then in TBS + 0.1% Tween (TBSTw) for 5 min (3 times). Embryos were then blocked in 10% heat-inactivated mouse serum in TBSTw for 2.5 h.

While embryos were in blocking solutions, the anti-digoxigenin antibody (Roche Diagnostics) was preabsorbed to reduce background staining. This was accomplished by incubating the antibody for 1 hour at a 1/1000 dilution in 2% blocking reagent (Boehringer Mannheim) + 1% of a 2mM levamisole solution + dried mouse embryo powder in TBSTw.

The embryos were then incubated in 50% preabsorbed antibody solution + 2mM levamisole + 1% heat-inactivated mouse serum (1/2000 final antibody dilution) at 4°C overnight. They were then thoroughly washed in 2mM levamisole in TBSTw. They were then transferred to alkaline phosphatase (AP) developing solution (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween-20, 2mM levamisole). Detection was carried out in 0.5% NBT and 0.375% BCIP (Promega) in AP developing solution. Once color detection was complete, embryos were thoroughly washed in 20mM EDTA in PBSTw, post-fixed in 4% paraformaldehyde and washed in PBSTw. They were then carried through a dehydration series into 100% methanol to clear background staining and then a rehydration series back into PBSTw.

The following molecular markers were used for mouse whole mount *in situ* hybridization: *Ipw*, *Pax1* and *Unc45a*. The *Ipw* and *Pax1* constructs were gifts from Dr. Heather McDermid, University of Alberta.

2.6.3 *In situ* hybridization of zebrafish embryos

Embryos of the desired stages were manually dechorionated and fixed in 4% paraformaldehyde overnight at 4°C. Following fixation embryos were washed in PBS + 0.1% Tween-20 (PBSTw). Embryos over 12 hpf were permeabilized with 10 µg/mL proteinase K in PBSTw at room temperature. 12-16 hpf embryos were treated for 1 min; 18 hpf embryos were treated for 3 min; 24 hpf embryos were treated for 5 min; 36 hpf embryos were treated for 8 min; 48 hpf embryos were treated for 15 min; 72 hpf embryos were treated for 20 min. Following permeabilization, embryos were fixed in 4% paraformaldehyde for 20 min at room temperature and washed with PBSTw.

Embryos were prehybridized at 70°C in hybridization solution (50% formamide, 5x SSC, 500 µg/mL yeast tRNA, 50 µg/mL heparin, 0.1% Tween-20, pH 6.0) for 2 h. Following prehybridization, embryos were hybridized with a 1/200 dilution of antisense RNA probe in hybridization solution at 70°C overnight.

Following hybridization, the remaining treatments were performed in a Leica EM TP tissue processor with the embryos placed in 8 well baskets specific to the machine agitating at 0.25 Hz. Embryos were washed at 60°C for 5 min in the following solutions: wash buffer (50% formamide, 1x SSC, 0.1% Tween-20), 2:1 wash buffer: 2x SSC, 0.2% Tween-20, 1:1 wash buffer: 2x SSC, 0.2% Tween-20, 1:2 wash buffer: 2x SSC, 0.2% Tween-20, 2x SSC, 0.2% Tween-20. The high stringency washes (0.05x SSC, 0.2% Tween-20) were performed at 60°C for 30 min (twice). The embryos were then washed in PBSTw for 5 min at room temperature and placed in blocking solution (40 mg/mL BSA + 5% goat serum in PBSTw) for 2 h at room temperature. Embryos were then

incubated with a 1/10 000 dilution of anti-digoxigenin antibody (Roche Diagnostics) in blocking solution for 15 h at 4°C. Following incubation with the anti-digoxigenin antibody, washes were all performed at room temperature with an agitation rate of 1 Hz. The embryos were washed in PBSTw for 15 minutes (5 times), in 0.05% Tween-20 for 5 minutes, and finally in NTMT (100 mM NaCl, 100 mM Tris-HCl pH 9.5, 50 mM MgCl₂, 0.05% Tween-20) for 10 minutes (4 times). Embryos were removed from the Leica EM TP machine before staining was initiated.

To develop the color reaction, embryos were placed in a 2% mixture of NBT/BCIP Stock Solution (Roche Diagnostics) in NTMT until staining reached a desired level. Embryos were then washed thoroughly in 0.05% Tween-20 and placed in 0.05% Tween-20 in methanol at 4°C at least overnight to remove background staining.

The following molecular markers were used for zebrafish *in situ* hybridizations: *myf5* (Coutelle et al., 2001), *myh6* (Berdougo et al., 2003), *myl7* (Yelon et al., 1999), *myod* (Weinberg et al., 1996), *vmhc* (Yelon et al., 1999), *unc45a* and *unc45b*. The *myf5* construct was a gift from Dr. Simon Hughes (Kings College London). The *myod* construct was a gift from Dr. Eric Weinberg (University of Pennsylvania). The *myh6*, *myl7* and *vmhc* constructs were gifts from Dr. Deborah Yelon (New York University Medical Center).

2.7 Developmental RT-PCR

Total RNA was prepared from one adult male and 50-60 embryos of the following stages: 1-2 cell, 128-256 cell, 4 hpf, 6 hpf, 10-11 hpf, 24 hpf, 36 hpf, 48 hpf, 3

dpf, 5 dpf, 7 dpf. 100 μ L of Trizol (Invitrogen) was added and the tissue was ground with a small plastic pestle. An additional 400 μ L of Trizol was then added, followed by a 5 min incubation at room temperature. 200 μ L of chloroform was added, the mixture was vortexed for 15 sec and left at room temperature for 3 min. This was centrifuged at 12,000 $\times g$ at 4°C for 15 min and the aqueous layer was collected. RNA was precipitated by adding 300 μ L of isopropanol and letting the mixture sit for 10 min at room temperature, followed by centrifugation for 10 min at 12,000 $\times g$ at 4°C. The pellet was washed in 70% ethanol in DEPC-treated water, centrifuged for another 5 min at 7,500 $\times g$ at 4°C, allowed to air dry and resuspended in 20-30 μ L of nuclease-free water.

cDNA was made with the iScript cDNA Synthesis Kit (BioRad) according to manufacturer's instructions using 1.5 μ g of total RNA as template.

PCR was performed using the primer pairs RT FOR1 and RT REV1 or Bactin FOR and Bactin REV with the following conditions: 94°C, 45 sec (94°C, 15 sec; 53°C, 30 sec; 68°C, 1 min) for 35 cycles. The Bactin primers were used as a positive control to amplify β actin cDNA. As a negative control, PCR was performed using RNA as a template for the RT FOR1 and RT REV1 primers.

2.8 Morpholino design and injection

Antisense morpholino oligonucleotides were designed and ordered from Gene Tools, LLC (Philomath, OR).

unc45b MO (1) (5' ATCTCCAATTCTCCCATCGTCATT 3') was designed complementary to bp 124-148 of the *unc45b* transcript which includes the translational

start. *unc45b* MO (2) (5' AGTCAATTCAGCTTCGTCGTAAGAG 3') was designed as a non-overlapping morpholino upstream of *unc45b* MO (1). *unc45b* MO (2) is complementary to bp 97-121 of the *unc45b* transcript. I also used an *unc45a* morpholino (5' ACATCTTTCTCTGTAGCGTTGTATT), complementary to bp 165-189 of the *unc45a* transcript. This morpholino was designed to cover the translational start site of *unc45a* transcript (gift from Dr. Beth Roman, Georgetown University). As a negative control, *zfdelta* MO (5' GACGTTGTCATTTATTTGATTTTCG 3'), a morpholino with no known complementary sequence in the zebrafish genome, was injected at the same dose as *unc45b* MO (1).

For injections, morpholinos were diluted in either dH₂O or Danieau buffer (Nasevicius and Ekker, 2000). They were injected using glass needles drawn from 1mm capillary tubes. 0.1-10 ng of morpholino was injected into the yolks of fertilized embryos between the 1 and 4 cell stage.

2.9 Construction of *unc45b*::GFP fusion

Nucleotides 54-492 of the *unc45b* cDNA (see Appendix IV) were fused upstream of a GFP coding region by amplifying this region using the UNC_N1_FOR and UNC_N1_REV primers with the following reaction conditions: 95°C, 30 sec (95°C, 15 sec; 49°C, 30 sec; 68°C, 3 min) for 30 cycles. This PCR fragment was digested with EcoR1 and BamH1, purified with the Sephaglas BandPrep Kit (Amersham) and ligated into EcoR1/BamH1-digested pEGFP N1 vector (Clontech). To allow *in vitro* transcription of the *unc45*::GFP fusion, the construct was digested with *EcoRI* and *NotI*

(to excise a fragment containing the *unc45b*-GFP fusion) and the appropriate fragment was purified using the Sephaglas BandPrep Kit (Amersham) and ligated into *EcoRI*/*NotI*-digested pBluescript SK.

The GFP coding region was also cloned into pBluescript SK. This region was amplified using the C1_GFP_FOR and C1_GFP_REV primers with the following reaction conditions: 95°C, 30 sec (95°C, 15 sec; 57°C, 30 sec; 68°C, 1 min) for 30 cycles. This PCR fragment was digested with *EcoRI* and *NotI*, purified with the Sephaglas BandPrep Kit (Amersham) and ligated into *EcoRI*/*NotI*-digested pBluescript SK.

Synthetic capped RNAs were produced from *NotI*-digested *unc45b*-GFP-pBluescript SK or GFP-pBluescript SK using the T7 mMessage mMachine kit (Ambion Inc.) and poly-A tails were added using the Poly(A) Tailing Kit (Ambion Inc.) according to manufacturers instructions. RNA was then dissolved in nuclease-free water. Embryos at the one-cell stage were injected with 0.2 ng of RNA with or without 2 ng *unc45b* MO(1). GFP expression was assessed at approximately the 75% epiboly stage.

2.10 Construction of Myc::*unc45b* fusion

To create an amino-terminus fusion protein consisting of 6 Myc epitope tags fused to Unc45b expressed behind a ubiquitous CMV promoter the CS3+MT vector was used (constructed by Anne Vojtek, University of Michigan). To amplify the coding region of *unc45b*, the primers CS3MT UNC FOR and CS3MT UNC REV were used with the following reaction conditions: 95°C, 30 sec (95°C, 15 sec; 55°C, 30 sec; 68°C, 3 min) for 30 cycles. The PCR fragment was purified using the Sephaglas BandPrep Kit

(Amersham) and cloned into the pGEM-T vector (Promega). This construct was then digested with *EcoRI* and *XhoI* and the insert was again purified using the Sephaglas BandPrep Kit (Amersham) and ligated into *EcoRI/XhoI*-digested CS3+MT vector.

Approximately 200 pg of this construct and 200 pg of pEGFP_C1 (Clontech) were injected into embryos at the one-cell stage. The pEGFP_C1 was used as a co-injection marker to facilitate screening for successfully injected embryos by looking for GFP expression in the trunk of the animals. These animals were then fixed and protein was detected using an anti-Myc antibody and an Alexa-546 fluorescent secondary antibody as described in section 2.11.1.

2.11 Immunostaining

2.11.1 Immunofluorescence

Embryos were fixed in 2% trichloroacetic acid for 2-3 h at room temperature, washed in PBS+0.1% TritonX-100 (PBSTx) and blocked overnight at 4°C in 5% bovine serum albumin in PBSTx. Embryos were incubated in an appropriate dilution of primary antibody [1/2500 dilution of anti-Myc, 1/10 dilution of F59, MF20, S46 or S58 (Developmental Studies Hybridoma Bank, Iowa City, IA)] in blocking solution overnight at 4°C and washed in PBSTx. Embryos were then incubated in secondary antibody [1/2500 dilution of anti-mouse Alexa 546 or 1/1000 dilution of anti-mouse Alexa 488 (Molecular Probes)] and washed in PBSTx before viewing.

2.11.2 DAB staining

Whole-mount cardiac immunostaining was performed using the monoclonal antibodies F59, MF20 and S46 (Developmental Studies Hybridoma Bank, Iowa City, IA). Embryos were fixed in 2% trichloroacetic acid for 2-3 h at room temperature, washed in PBS+0.1% TritonX-100 (PBSTx) and blocked for 2 h at room temperature in 5% bovine serum albumin in PBSTx. Embryos were incubated in a 1/10 dilution of primary antibody in blocking solution overnight at 4°C and washed in PBSTx. Embryos were then incubated in a 1/300 dilution of horseradish peroxidase-conjugated anti-mouse secondary antibody (Amersham Biosciences) for 4 h at room temperature and washed in PBSTx. Finally embryos were then treated with 1 mL of 0.5% DAB (diaminobenzidine), 1% DMSO in PBS for 15 min at room temperature. 2.5µL of 3% H₂O₂ was added to develop stain. Once a desired level of staining was achieved, embryos were washed in PBSTx to stop staining before viewing.

2.12 Phalloidin staining

48 hpf embryos were fixed in 4% paraformaldehyde for 1 h at room temperature and were washed in PBS+ 0.1% Triton X-100 (PBSTx). Embryos were incubated in a 1/50 dilution of AlexaFluor 568 phalloidin (Molecular Probes) in PBS for 30 min at room temperature. Embryos were then washed in PBSTx, mounted in 3% methylcellulose and viewed on a Leica DM/RBI confocal laser scanning microscope.

2.13 Transmission electron microscopy

48 hpf embryos were fixed in 2.5% glutaraldehyde in PBS overnight at 4°C. They were then washed in PBS and transferred to a Leica EM TP tissue processor in 8-well baskets where they underwent the following treatments: (PBS, 10 min) 3 times; 2% osmic acid, 2 h; PBS, 10 min; 20% ethanol, 10min; 30% ethanol, 10min; 40% ethanol, 10min; 50% ethanol, 10min; 60% ethanol, 10min; 70% ethanol, 10min; 80% ethanol, 10min; 90% ethanol, 10min; absolute ethanol, 10min; 50% propylene oxide/50% ethanol, 10 min; propylene oxide, 10 min.

Embryos were taken out of the tissue processor and transferred to a rubber mold with 50% propylene oxide, 50% Spurr resin which was then replaced with 100% Spurr resin. To solidify the resin, they were left overnight at room temperature, 30°C for 3 h and 60°C overnight. Excess plastic and tissue were trimmed with a razor blade. Ultrathin (60-70 nm) sections of the trunk were cut on a Reichert-Jung Ultracut E. Sections were stained with uranyl acetate for 1 h, followed by lead citrate for 1 min and viewed on Philips Morgagni 268 transmission electron microscope.

2.14 Alcian blue staining

5 dpf embryos were fixed overnight at 4 °C in 4% paraformaldehyde and washed in PBS. They were then rinsed in 80% ethanol, 20% glacial acetic acid and stained overnight at room temperature with 0.1% Alcian Blue 8GX (Sigma) in 80% ethanol/20% glacial acetic acid. Embryos were then rehydrated to PBS through a series of 5 min washes (80% ethanol, 60% ethanol, 40% ethanol, 20% ethanol, PBS) and treated with

0.05% trypsin in saturated sodium tetraborate for 2 h. Pigmentation was removed by treatment with fresh 3% hydrogen peroxide, 1% potassium hydroxide for 8 h. Embryos were then washed in PBS and photographed.

2.15 Expression of GST::Unc45b in *E. coli*

Nucleotides 104-2979 of the *unc45b* cDNA (see Appendix IV) encoding the entire Unc45b protein were cloned downstream of a glutathione S-transferase (GST) coding region by amplifying this region using the ZF UNC45B FOR2 and ZF UNC45B REV2 primers with the following reaction conditions: 94°C, 30 sec (94°C, 15 sec; 68°C, 20 sec - 1°C/cycle; 68°C, 5 min) for 10 cycles, followed by (94°C, 15 sec; 58°C, 20 sec; 68°C, 5 min + 6 sec/cycle) for 25 cycles. This PCR fragment was digested with *EcoRI*, purified with the Sephaglas BandPrep Kit (Amersham) and ligated into *EcoRI*-digested, phosphatase-treated pGEX 1λT vector (Amersham).

Additionally a smaller region of the *unc45b* cDNA (nucleotides 2-404, encoding amino acids 2-136 of the Unc45b protein) was cloned downstream of a GST coding region. This region was used because it was predicted that the amino-terminus of Unc45b would be more soluble than the full-length protein based on findings from *C. elegans* UNC-45 (Ao, 2001; Horlick, 2005). This region was amplified using the ZF PGEX2T FOR and ZF PGEX2T REV1 primers with the following reaction conditions: 95°C, 30 sec (95°C, 15 sec; 53°C, 30 sec; 68°C, 3 min) for 30 cycles. The PCR fragment was digested with *EcoRI* and *BamHI* and purified with the Sephaglas BandPrep Kit (Amersham) and ligated into *EcoRI/BamHI*-digested pGEX 2T vector (Amersham).

These constructs were transformed into competent *E. coli* BL21 (*DE3 pLysS*) cells (Stratagene) to allow expression of these proteins. Cells were grown at 37°C in 1 L of 2xYT media to an OD₆₀₀ of approximately 0.6, followed by induction of expression by the addition of 1 mL of 1M IPTG. Cells were then placed back at 37°C with agitation for an additional 3 h before being pelleted.

Recombinant proteins were purified according to manufacturer's instructions. Briefly, bacterial pellets were resuspended in PBS and lysed by French press at 1500 psi 4 times. The cellular material was then incubated with Triton X-100 at a final concentration of 1% for 30 min. Cellular debris was pelleted and the supernatant was incubated with a slurry of Glutathione Sepharose 4B (Amersham) for 30 min. Beads were repeatedly washed with PBS to remove unbound proteins. Recombinant protein was eluted with 10mM reduced glutathione in 50 mM Tris-HCl, pH 8.0. Eluate was then dialysed into PBS and concentrated using Amicon Ultra Centrifugal Filter Devices (Millipore).

2.16 Antibody production and purification

Polyclonal antibodies were raised against GST::Unc45b by first immunizing rabbits 10-9 and 12-5 with approximately 750 µg of recombinant protein in Freund's complete adjuvant (Sigma). Rabbits were injected an additional 4 times with 750 µg of recombinant protein in Freund's incomplete adjuvant (Sigma) at 3-4 week intervals. Test bleeds were collected 2 weeks after each injection to check for presence/titre of antibody. Following exsanguinations, serum was aliquoted and stored at -80°C.

In an attempt to clear antisera of antibodies raised against general *E. coli* BL21 proteins serum was incubated with a powder made from acetone-dehydrated *E. coli* BL21 cells at a final concentration of 1%. The supernatant of this slurry was used to test reactivity of the remaining antibodies.

Alternatively, antisera was purified using nitrocellulose membrane bound with large quantities of Unc45b (transferred by electroblotting). This membrane was blocked overnight in 3% BSA in PBS, washed 3 times in PBS, and then incubated with a mixture of 20% antisera in PBS. Following incubation, the membrane was collected and washed in PBS. Antibodies recognizing GST::Unc45b were eluted by incubating the nitrocellulose with 1 mL 0.1M glycine, pH 2.3 for 2 min, followed by neutralization in 90 μ L 1M Tris-HCl, pH 8.8 and 20 μ L 5M NaCl. Antibodies were then dialyzed into PBS and concentrated using Amicon Ultra Centrifugal Filter Devices (Millipore).

As a final means of purifying the antisera, an affinity column was employed subsequent to binding of GST::Unc45b or GST::Unc45b (aa 2-404) proteins to Sepharose. Purified proteins were coupled to CNBr-activated Sepharose 4 Fast Flow (Amersham) according to manufacturer's directions. A 20% solution of antisera in PBS was incubated with the affinity column overnight at 4°C. The Sepharose was then washed for 5 min in PBS (3 times). Purified antibodies were eluted from affinity column with the addition of 1 mL 0.1M glycine, pH 2.3 for 2 min as above, followed again by immediate neutralization in 90 μ L 1M Tris-HCl, pH 8.8 and 20 μ L 5M NaCl. Finally purified antibodies were dialyzed into PBS and concentrated using Amicon Ultra Centrifugal Filter Devices (Millipore).

2.17 Testing reactivity and specificity of antisera

2.17.1 Bacterial and zebrafish lysate

Bacterial cultures were pelleted and resuspended in an approximately equal volume of 2 x Laemmli SDS-PAGE loading buffer. Cells were then incubated in boiling water for approximately 5 min, cellular debris was pelleted and the supernatant collected.

Zebrafish lysates were prepared by pelleting embryos and flash freezing using liquid nitrogen. Embryos were then ground using a plastic pestle in an approximately equal volume of 2 x Laemmli SDS-PAGE loading buffer. Lysate was incubated in boiling water for approximately 5 min and, again, the cellular debris was pelleted and the supernatant collected.

2.17.2 Western analysis

Proteins were separated on 10-14% SDS-PAGE gels and electroblotted onto nitrocellulose at 90 volts for 1 h. Membranes were stained with Ponceau S to ensure transfer was successful then blocked overnight at 4°C using 5% skim milk powder in TBSTw. Membranes were then incubated with primary antibodies at dilutions ranging from 1/250-1/5000 for 1 h at room temperature. The western blot was then washed for 15 min in TBSTw (4 times), incubated with a 1/10 000 dilution of anti-rabbit horseradish peroxidase linked secondary (Amersham) for 30 min at room temperature then washed again for 15 min in TBSTw (4 times). Antibody reaction was detected using SuperSignal West Pico Chemiluminescent substrate (Pierce) according to manufacturer's directions.

2.18 Identification of *herzschlag*, *jam* and *still heart* heterozygotes

The progeny of a cross between a *herzschlag* (*hel*^{tg287}), *jam* (*jam*^{tr254a}) or *still heart* (*sth*^{tm123a}) heterozygote with the wildtype Tübingen (Tu) strain were received from the Max-Planck-Institut für Entwicklungsbiologie (Tübingen, Germany). From these crosses, approximately half of the fish should have been heterozygous for the mutation of interest; the other half should have been phenotypically wildtype but would carry the same background mutation as the heterozygotes. At sexual maturity, to identify adult heterozygotes, matings of two fish at a time were set up and the progeny collected. The pairs of mated fish were then placed in small tanks until genotypes were determined. The embryos were examined at 24 hpf and 48 hpf for motility and cardiac defects which would indicate that both parents were heterozygous for the mutation of interest.

2.19 Sequencing of *unc45b* from *herzschlag* and *still heart* homozygotes

Heterozygotes were crossed and progeny were collected. Embryos were examined between 26 and 59 hpf and the homozygous progeny (embryos displaying motility defects) were collected, frozen in liquid nitrogen and stored at -80°C. RNA was extracted as described in section 2.7.

cDNA was synthesized with Superscript III Reverse Transcriptase (Invitrogen) according to manufacturer's instructions using 400 ng of adapter primer (Gibco) and 2 pmol of REV3783_full (*unc45b*-specific primer) with approximately 2 µg of RNA as template.

PCR was performed (in duplicate) to amplify the *unc45b* coding region using the primers ZF PGEX2T FOR and ZF PGEX2T REV7 with the following reaction conditions: 95°C, 30 sec (95°C, 15 sec; 63°C, -1°C /cycle, 30 sec; 68°C, 3 min) for 10 cycles, (95°C, 15 sec; 53°C, 30 sec; 68°C, 3 min + 6 sec/cycle) for 25 cycles. PCR was also used to amplify the 5'UTR of the *unc45b* cDNA using the FOR26_full and ZF PGEX2T REV3 primers with the following reaction conditions: 95°C, 30 sec (95°C, 15 sec; 50°C, 30 sec; 68°C, 1 min) for 30 cycles. The PCR fragments were purified using E.Z.N.A. Poly-Gel DNA Extraction Kit (UBI) and sequence was obtained directly from the duplicate PCR products to mask PCR-induced mutations.

To identify coding changes, sequences were aligned with the wildtype gene sequence using the CAP Sequence Assembly Machine program (<http://bio.ifom-firc.it/ASSEMBLY/assemble.html>).

2.20 Photography and image processing

All embryos were mounted in 3% methylcellulose for viewing. DIC and fluorescent illumination were viewed on a Zeiss Axioskop 2 mot *plus* compound microscope and photographed using a Retiga EXi camera. Live 24 hpf-5 dpf embryos, *in situ* hybridizations, DAB stained and alcian blue stained embryos were observed with an Olympus SZ X12 dissecting microscope. Images were captured using a Qimaging MicroPublisher 3.3 RTV digital camera. Rhodamine-phalloidin stained embryos and fluorescent immunohistochemistry was viewed and photographed on a Leica DM/RBE

confocal laser scanning microscope. Images were processed using Adobe Photoshop 7.0 or Adobe Photoshop CS.

3. Results

Please note that when referring to the *unc45* gene or protein that it is the zebrafish homologues being discussed unless otherwise stated.

3.1 Comparison of vertebrate *unc-45* homologues

For a number of years *unc-45* has been studied in invertebrates (Ao, 2001; Checkland, 2004; Horlick, 2005) but at the onset of my project it had never been characterized in a vertebrate system. In order to determine if the role of UNC-45 is conserved across metazoans its function had to be examined using other model organisms. To accomplish this, homologues of the *C. elegans unc-45* gene first had to be identified. This was accomplished by performing database searches using the 961 amino acid *C. elegans* UNC-45 as the starting point. Vertebrate homologues were recognized based on high levels of identity between their predicted proteins and *C. elegans* UNC-45. The percentage of identity measures the percentage of amino acids at the same position that are identical between two proteins while the percentage of similarity also includes amino acids with similar characteristics, such as amino acids that are acidic or basic. These values help predict if two proteins are related and, if so, how closely related they are. As expected, each homologue identified encoded a protein with domains organized in a similar pattern as *C. elegans* UNC-45. Each is composed of an amino terminal TPR domain, a central domain that does not carry any similarity to proteins other than other UNC-45 homologues and a carboxyl terminal UCS domain.

The first homologue I identified was the *Homo sapiens UNC45A* homologue. This gene resides on chromosome 15 and encodes a cDNA which is 3192 basepairs long which results in a 944 amino acid protein that has 31% identity to *Caenorhabditis elegans* UNC-45 at the protein level. The *Mus musculus* homologue is 3264 basepairs (residing on chromosome 7) and also encodes a 944 amino acid protein that is 94% identical/97% similar to its human counterpart. It, therefore, is also 31% identical to *C. elegans* UNC-45 at the amino acid level (Figure 3.1). Following my identification of these homologues, Price *et al.* (2002) published a study involving these human and mouse *UNC45A* homologues and also revealed that there are two *UNC45* homologues in mammals. This second homologue is termed *UNC45B* and is located on human chromosome 17 and mouse chromosome 11. Each *UNC45B* protein is 55% identical and 75% similar to the *UNC45A* homologues in their respective species (Price *et al.*, 2002).

The *Danio rerio unc45a* homologue is found on zebrafish chromosome 25 and encodes a 936 amino acid protein that is 65% identical to its mammalian homologues, while only 55% identical to mammalian *UNC45B* and 33% identical to *C. elegans* UNC-45 at the protein level (Figure 3.2). *Unc45a* is 55% identical to the zebrafish *Unc45b* protein. The conservation of these proteins is evenly distributed across the full length of the protein and do not show any extended region of conservation (Figure 3.3). The *unc45b* gene is located on chromosome 8. The 934 amino acid *Unc45b* protein is 71% identical to mammalian *UNC45B* proteins and only 32% identical to the UNC-45 protein in *C. elegans* (Figure 3.2, Figure 3.4). As discussed earlier UNC-45 proteins share a domain (the UCS domain) with proteins found in fungi. The UCS domains of the

zebrafish *Unc45a* and *Unc45b* proteins are respectively 21% and 20% identical to the UCS domain of She4p so, expectedly, the conservation is lower when comparing identity across kingdoms than when comparing different animal species (Figure 3.4).

While the *unc45a* and *unc45b* cDNAs are only 3315 basepairs and 3848 basepairs, respectively (the difference in length due mainly to differences in the length of their 3' UTRs) they respectively cover 33673 and 25302 basepairs of genomic sequence. Each gene is encoded within 20 exons and, from comparing the *unc45a* and *unc45b* genes it can be seen that the length and organization of these exons appear to have been maintained throughout evolution, with few exceptions (Figure 3.5, Figure 3.6, Table 3.1, Table 3.2).

Finally, although the zebrafish genome underwent a partial duplication following the divergence of the teleost and tetrapod lineages 420 million years ago, these are the only *unc45* homologues that exist in zebrafish (Amores et al., 1998). Currently there are no EST's or any genomic sequence that would indicate the occurrence of any zebrafish *unc45* homologue other than *unc45a* and *unc45b*. Similarly, there are no additional *Unc45* homologues in mammals. It appears that all vertebrate genomes contain two *unc45* homologues but invertebrate genomes, including that of the ascidian, *Ciona intestinalis* (thought to be an approximation of ancestral chordates) only contain one *unc45* gene.

3.2 Expression of mammalian *UNC45A*

3.2.1 Human Northern analysis

To determine in which tissues *unc45a* is expressed in *Homo sapiens*, I performed northern analysis using a human fetal and a human adult multi-tissue northern blot. Both northern blots showed a single band of approximately 3.2 kb when probed with radiolabelled human *UNC45A* cDNA, consistent with the expected size determined *in silico* (Figure 3.7). Because this was done prior to the publication that revealed the existence of two separate mammalian *UNC45* homologues (Price et al., 2002), I expected this gene to be expressed only in muscle-containing tissues, similar to *unc-45* in *C. elegans*. However expression was actually apparent in all tissues examined with slightly higher expression in the adult kidney, placenta and skeletal muscle. It should be noted that the three bands observed in the heart and skeletal muscle lanes when probed with β *actin* (Figure 3.7C, D) are due to multiple β *actin* isoforms present in these tissues.

3.2.2 Mouse *in situ* hybridization

Although northern analysis demonstrated that human *UNC45A* is expressed in numerous tissue types, the categorization of tissue type used for these northern blots was quite broad. Therefore I performed *in situ* hybridization on mouse embryos using an antisense murine *Unc45a* probe composed of the entire cDNA (Figure 3.8) with hopes of visualizing *unc45a* expression more specifically. At 11.5 dpc, there is no strong staining pattern observed though there is deeper staining in the branchial arches and limb buds (Figure 3.8A, B). At 14 dpc the highest expression levels are seen in the brain, limb buds and tail (Figure 3.8E, F). The positive control *in situ* hybridizations with *Pax1* and *Ipw* provide the expected expression patterns (somites/limb bud and brain/lateral to midline,

respectively) and demonstrate that the technique was successful (Figure 3.8C, G). The lack of color development in the negative control indicates that the staining observed in the experimental embryos is beyond background levels and should therefore be considered significant (Figure 3.8D, H).

Although mice are a useful model organism zebrafish possess many advantages in the study developmental processes. Zebrafish embryos are plentiful, transparent and develop externally so we can observe their development from start to finish. Additionally morpholino technology makes it simple to knockdown protein levels of a given gene thereby easily phenocopying a mutant phenotype. After considering these advantages, I decided to continue my project by studying the role of *unc45* in zebrafish.

3.3 Expression of *unc-45* in zebrafish

3.3.1 *In situ* hybridization

3.3.1.1 *unc45a*

To determine the expression pattern of *unc45a* in *Danio rerio*, I performed *in situ* hybridization on 26 hpf and 48 hpf zebrafish embryos using the *unc45a* 3' UTR as a probe to reduce any potential cross hybridization with the *unc45b* homologue (Figure 3.9). At both stages examined there is nearly ubiquitous staining observed, with possible enrichment in the vascular system.

3.3.1.2 *unc45b*

Given that postembryonic *C. elegans unc-45* and mammalian *Unc45b* expression is limited to muscle tissue, we expected the same of *unc45b* in zebrafish (Price et al., 2002; Venolia et al., 1999). To reduce potential cross-hybridization with any *unc45a* transcript, expression of *unc45b* was detected by whole mount *in situ* hybridization with an antisense probe corresponding to the 3' UTR of the *unc45b* coding region (Figure 3.10). As reported in Etheridge *et al.* (2002) *unc45b* expression originates at around 9 hpf in the adaxial cells (slow muscle progenitor cells) and is observed in presomitic mesoderm beginning at 10 hpf, continuing until the completion of somitogenesis. The most distinct expression is observed in the somites where it can be seen until approximately 72 hpf. In addition to the trunk muscle expression, *unc45b* expression can be detected in the pectoral fin muscle at 48 hpf and craniofacial muscle at 3dpf (Figure 3.11).

unc45b is also expressed in the cardiac mesoderm with expression initiated at 18 hpf in bilateral stripes of cells that will fuse to become the heart tube (Figure 3.12). At 24 hpf *unc45b* is detectable in both the atrium and ventricle of the developing heart however atrial expression is reduced by 36 hpf and no longer detectable by 44 hpf.

3.3.3 RT-PCR

Although *unc-45* transcript is maternally provided in *C. elegans* and expressed through adulthood (Venolia et al., 1999; Venolia and Waterston, 1990), the *in situ* hybridization did not reveal any expression of *unc45b* in zebrafish before 9 hpf or past 72 hpf at the transcriptional level. Due to its higher sensitivity, RT-PCR was performed to

address whether *unc45b* is present at stages where *in situ* hybridization was unable to detect any expression (Figure 3.13). Using this technique it is observed that *unc45b* is maternally provided as evidenced by the positive result observed when using *unc45b*-specific primers on cDNA from 1-2 cell embryos. *unc45b* transcript is present in all stages examined, including adult males indicating that *unc45b* is continually expressed. β actin-specific primers were used as a positive control to ensure that each sample contains intact cDNA. The negative controls lanes do not contain any product with the exception of the 36 hpf stage. However, because significant *unc45b* expression is observed at both 24 hpf and 48 hpf, where the negative control lanes did not exhibit contamination, it is reasonable to conclude that the *unc45b* expression observed at 36 hpf is not an artifact of contamination.

3.4 Protein localization (Myc::Unc45b fusion)

Considering the expression of *unc45b* is exclusively in striated muscle in zebrafish embryos and that immunostaining revealed that UNC-45 is localized to the thick filaments of body wall muscles in *C. elegans* (Ao and Pilgrim, 2000) we expected that the Unc45b protein would also localize to thick filaments in zebrafish embryos. Because we lacked a functional anti-Unc45b antibody I attempted to examine the localization of Unc45b in zebrafish using a construct encoding a version of Unc45b fused with six Myc epitopes. This construct was co-injected with a GFP-encoding construct into embryos at the one cell stage. Screening for GFP expression identified live embryos expressing the fusion protein in their trunk muscle. Immunostaining was then performed

on these embryos using an anti-Myc antibody (Figure 3.14). Although the Myc::Unc45b fusion is expressed within muscle cells as evidenced by the positively stained multinucleated cells spanning the width of the somite, no defined localization to the sarcomeres is observed. Instead the fusion protein appears to be present only in the cytoplasm in these cells. We cannot conclude that this would be the endogenous localization of this protein however as it may be the result of misfolding, and therefore mislocalization, of the protein due to the addition of the Myc epitopes onto the Unc45b protein.

3.5 Phenotype of *unc45b* morphants

In the absence of a genetic lesion in the *unc45b* gene, antisense morpholino oligonucleotides were used to reduce protein levels. To test the requirement for Unc45b in zebrafish muscle development and function, I designed two non-overlapping morpholino oligonucleotides (MO's), one targeted to the translation start site of the mRNA and the other to the region upstream of the translation start site (Nasevicius and Ekker, 2000). To ensure that the observed morpholino-mediated effects were in fact due to reduction in the amount of Unc45b, I tested the ability of *unc45b* MO (1) to block translation of a GFP reporter. One cell-staged embryos were injected with RNA encoding the 5' untranslated region and the first 123 amino acids of *unc45b*, which includes the morpholino binding site of *unc45b* MO (1), fused to a GFP-encoding region (*unc45b::GFP*). Injection of this RNA along with *unc45b* MO (1) resulted in the absence of detectable GFP (compare Figure 3.15B and D). However the injection of morpholino

had no effect on the expression levels of GFP if the morpholino binding site is absent (compare Figure 3.15F and H). In addition to the similarity of the phenotypes observed by injection of the two morpholinos, this demonstrates the specificity of the *unc45b* morpholinos.

Neither morpholino produces any gross defects in development at 24 hpf with the exception of severe paralysis (Figure 3.16, Figure 3.17). Injection of 5 ng of either morpholino results in this paralysis (*unc45b* MO (1) = 98%, n=124; *unc45b* MO (2) = 86.6%, n=134) (Table 3.3, Figure 3.17), consistent with the phenotype of *C. elegans unc-45(r450ts)* mutants raised at the restrictive temperature (Epstein and Thomson, 1974). At 48 hpf, morphants lack ventricular pumping (*unc45b* MO (1) = 96%, n=76; *unc45b* MO (2) = 61%, n=125) and blood circulation (*unc45b* MO (1) = 96%, n=76; *unc45b* MO (2) = 93%, n=125) (Table 3.3). The percentage of embryos lacking circulation is higher than those lacking ventricular pumping because in those embryos that possess a functional ventricle, the timing or strength of the contractions is impaired. Erythropoiesis appears to remain unaffected as non-circulating blood cells can be observed in the heart of affected embryos. In addition to the cardiac dysfunction, morphants often display cardiac edema initiating at 48 hpf (Figure 3.16). Both morpholinos resulted in increased severity of phenotypes with increased dose but because *unc45b* MO (1) produced the most severe effect at the highest penetrance (see Table 3.3), all further experiments were conducted using 5 ng of this morpholino to reduce potential morpholino toxicity.

To test if the effects of reducing Unc45b levels were worsened by also reducing Unc45a levels, morpholinos directed against both the *unc45a* transcript and the *unc45b*

transcript were co-injected. However these embryos do not display any phenotypes more severe than those injected with the *unc45b* MO (1) alone (data not shown).

3.6 Myogenic markers in *unc45b* morphants

UNC-45 has been implicated in the early stages of myocyte differentiation in tissue culture (Price et al., 2002) therefore I examined several muscle differentiation markers in our morphants to test whether myogenesis is affected *in vivo*. If the expression of these markers were downregulated or eliminated, it would indicate that UNC45B is upstream of the myogenic switch in cell differentiation. Whole-mount *in situ* hybridization against the myogenic regulatory factors, *myf5* and *myod*, was performed (Figure 3.18). However, the expression pattern of both *myf5* and *myod* is unaltered in *unc45b* morphants therefore it can be assumed that, in contrast to the results in cell culture, myogenesis is unperturbed in these embryos.

3.7 Skeletal muscle organization in *unc45b* morphants

In *C. elegans unc-45* mutants, the thick filaments in body wall muscles are disorganized (Epstein and Thomson, 1974; Venolia and Waterston, 1990). I examined the structure of the trunk muscle in morphants to determine if this was also the case in zebrafish. Observation under differential interference contrast (DIC) optics shows a clear difference between the muscle structure of control MO-injected embryos and *unc45b* MO (1)-injected embryos. The trunks of control embryos show the expected muscle striations. There is a lack of birefringency indicating absence of striations when Unc45b

levels are reduced (Figure 3.19A, B). At 5 dpf the somites of *unc45b* MO (1)-injected embryos become filled with vacuoles presumably due to tissue necrosis due to lack of circulation and/or functional sarcomeres (Figure 3.19C, D). Similarly, staining with fluorescently-labeled phalloidin, which labels the actin-containing thin filaments, also demonstrates proper organization of muscle structure in control embryos but not morphants (Figure 3.20).

Transmission electron microscopy (TEM) suggests that morphants lack myosin filaments in their trunk muscle; thin filaments are still present (Figure 3.21). In control embryos, the sarcomeres appear as regular chain-forming units resulting in functional myofibrils. Sarcomeres in *unc45b* morphants have a more jagged appearance with the middle region being void of any filaments at all. Therefore the paralysis of embryos with reduced levels of Unc45b is likely due to a resultant reduction in the numbers of myosin filaments and thereby myofibrils.

3.8 Slow muscle myosin organization in morphants

C. elegans unc-45 mutants have reduced levels of the major isoform of myosin heavy chain MHC B but not the minor isoform, MHC A (Barral et al., 1998). I performed immunostaining with myosin-specific antibodies to see if any myosins are grossly affected by blocking Unc45b translation in zebrafish. The monoclonal antibodies F59 and S58 both recognize slow-muscle myosin epitopes in zebrafish but the identity of their epitopes has not yet been determined (Crow and Stockdale, 1986; Devoto et al., 1996). In control 24 hpf embryos, both antibodies indicate a striated pattern of staining

as expected but morphants again lack this striation, only having weak, diffuse staining (Figure 3.22A-D). At 48 hpf, F59 stains wide stripes in each sarcomere in control embryos (Figure 3.22E). This is also the case in *unc45b* morphants although these embryos still lack motility. However, in these animals, the structure appears less compact and each myofibril is interrupted by what appears to be the muscle cell's single nucleus (Figure 3.22F). The myosin recognized by the S58 antibody has a much narrower staining pattern in each sarcomere than that detected by F59 (Figure 3.22G) possibly indicating a myosin arrangement similar to that of MHC A and MHC B seen in *C. elegans* muscle. In *C. elegans*, MHC A is only present in the 1.8 μm central region of the thick filament and MHC B is present in all but the central 0.9 μm of the thick filament, with a small region of overlap (Miller *et al.*, 1983). Unlike the results seen in F59 staining, 48 hpf morphants do not display any structure when stained with S58 (Figure 3.22H) indicating that this epitope is more strongly affected than the F59 epitope when UNC45B levels are reduced.

3.9 Cardiac abnormalities in morphants

3.9.1 *In situ* hybridization

After observing the lack of ventricular function in *unc45b* morphants I attempted to characterize the cardiac defects using *in situ* hybridization. To test whether the cardiac chamber identity is affected in *unc45b* morphants, I used *myh6* (previously known as *atrial myosin heavy chain*), *vmhc* (*ventricular myosin heavy chain*) and *myl7* (previously

known as *cardiac myosin light chain 2*) as molecular markers of chamber differentiation (Berdougo et al., 2003; Yelon et al., 1999). The expression patterns of *myh6* and *vmhc* are identical between controls and *unc45b* morphant embryos suggesting that the differentiation of the atrial and ventricular fates is unaffected when Unc45b levels are reduced and both heart-specific myosin heavy chains are expressed normally, at least at the transcriptional level (Figure 3.23A-D). *myl7* is expressed in both the atrium and ventricle, normally with distinct ventricular enrichment (Figure 3.23E). As observed in *wea* embryos and *tbx20* morphants, it appears that *myl7* levels are upregulated in the atrium of *unc45b* morphants to nearly those seen in the ventricle (Figure 3.23F) (Berdougo et al., 2003; Szeto et al., 2002).

3.9.2 Immunostaining

To examine if the pattern of protein expression is altered in the developing heart in response to the reduction of Unc45b levels immunostaining was performed using the S46, MF20 and F59 monoclonal antibodies (Bader et al., 1982; Crow and Stockdale, 1986). S46 recognizes an epitope of atrium-specific myosin heavy chain, Myh6 (Berdougo et al., 2003). The S46 staining pattern is unchanged in *unc45b* morphants compared to control embryos (Figure 3.24I-L) supporting the idea that the atrial myosin heavy chain expression is normal at the protein level as well as the transcriptional level (see section 3.9.1). As is seen with *myl7*, myosin heavy chain epitopes recognized by MF20 and F59 (Bader et al., 1982; Crow and Stockdale, 1986; Stainier and Fishman, 1992) are normally expressed in both chambers, with higher levels seen in the ventricle (Figure 3.24A-H). Again, in *unc45b* morphants, the levels of these proteins are nearly

identical in both chambers indicating their expression is upregulated in the atrium of morphants (Figure 3.24B, D, F, H).

3.9.3 Improper cardiac morphogenesis and edema

During cardiac morphogenesis in zebrafish, looping of the heart tube occurs at approximately 24 hpf, when the atrium jogs to the left of the ventricle. By 48 hpf, the ventricular region normally displays a distinct rightwards bend. Photography of a wildtype heart requires two planes of focus to capture both ventricle and atrium; hearts of *unc45b* morphants only require one plane of focus to be photographed (Figure 3.25A-D) suggesting improper cardiac looping. Use of the *myl7* *in situ* probe or the MF20 and F59 monoclonal antibodies as markers allows visualization of this looping. These markers obviously demonstrate that *unc45b* morphants fail to undergo cardiac looping (Figure 3.23, Figure 3.24). Instead the heart maintains a tube-like morphology despite proper molecular demarcation of the two chambers.

At approximately 48 hpf, pericardial edema is common in morphants; severe yolk-sac edema then develops at approximately 5 dpf (Figure 3.16, Figure 3.25F). Pericardial edema is never observed in control-injected embryos but occurs in *unc45b* morphants and other morphants or mutants with cardiac defects (Chen et al., 1996; Granato et al., 1996).

3.10 Effects of *Unc45b* knockdown on jaw structure

It was clear from morphant analysis that cranial structure abnormalities developed in morphants that were not observed in control animals. On a gross level, *unc45b* morphants display a dysmorphic jaw structure (Figure 3.26A, D). This, in addition to the observation that *unc45b* is also expressed in cranial muscle in the developing embryo (Figure 3.11B), prompted me to examine jaw cartilage organization using Alcian blue. Alcian blue staining of the cartilaginous structures in the head demonstrates that, while all the cartilages appear to be present in 5 dpf morphants, the Meckel's, palatoquadrate and basihyal cartilages are shorter and protruding and the basihyal, ceratohyal, basibranchial and ceratobranchial cartilages are displaced ventrally or "fallen" and protruding (compare Figure 3.26B, C and E, F). Although muscle function has not previously been implicated in craniofacial morphogenesis, this phenotype indicates a novel function of *unc45b* in the development of this region in zebrafish, presumably through a defect in muscle contraction.

3.11 Antibody Production and Purification

In an attempt to raise a specific anti-*Unc45b* antibody, I expressed a fusion protein consisting of glutathione S-transferase (GST) fused to the entire *Unc45b* protein in *E. coli* BL21 (*DE3 pLysS*) cells which was used to raise polyclonal antibodies in rabbits 10-9 and 12-5. The full length purified recombinant protein is the expected size (approximately 131 kDa) but is degraded within the bacteria (Figure 3.27A). These antisera consistently react against the recombinant GST::*Unc45b* protein (Figure 3.27B)

however when tested for reactivity against zebrafish lysate numerous bands are observed indicating that the crude antisera contains antibodies that react to proteins other than Unc45b or that any Unc45b is unstable and is also degraded into smaller cross-reacting peptides (Figure 3.27C). Numerous methods were employed to improve the specificity of the antisera. The antisera was incubated with an *E. coli* BL21 (*DE3 pLysS*) acetone powder to eliminate antibodies against *E. coli* proteins. This did reduce the number of proteins the antisera reacted against but no band was observed that was consistent with the size of Unc45b (103 kDa) (Figure 3.27D). Purification was also attempted by incubating the crude antisera with nitrocellulose bound with a large amount of GST::Unc45b protein followed by elution of the antibodies that react to this protein and through the usage of a GST::Unc45b affinity column. Although the purified antibodies do react strongly with the recombinant protein (Figure 3.27E, G) but as they do not recognize any 103 kDa protein (the expected size of Unc45b), they do not appear to react against zebrafish Unc45b (Figure 3.27F, H, I).

3.12 Phenotypes of mutants

In an attempt to identify a strain of zebrafish containing a mutation in the *unc45b* locus three strains of zebrafish, *herzschlag*, *still heart* and *jam* were obtained that, by preliminary characterization, display cardiac and motility phenotypes similar to those of *unc45b* morphants (Chen et al., 1996). The phenotypes of these strains are expanded upon below.

3.12.1 *herzschlag*

At 24 hpf homozygous *herzschlag* embryos display severely reduced motility. The only mobility they display is slight movement of their tails. This immotility worsens with age resulting in complete paralysis by 72 hpf. They lack circulation due to the weakness of the heartbeat and develop cardiac and yolk sac edema by 48 hpf (Figure 3.28). This phenotype worsens as they age and eventually homozygosity at this locus is lethal beginning at approximately 6 dpf.

3.12.2 *still heart*

Still heart embryos are completely paralyzed at 24 hpf however by 48 hpf they gain some motility, albeit weak. As the mutation name implies, the hearts of these embryos are completely nonfunctional. Like *herzschlag* embryos, *still heart* homozygotes develop severe cardiac and yolk sac edema, initiating at approximately 48 hpf (Figure 3.29). The edema observed in these embryos becomes increasingly severe and lethality is observed in the larvae beginning at approximately 4 dpf.

3.12.3 *jam*

Homozygous *jam* embryos are less severely affected than either *herzschlag* or *still heart* embryos. Their motility is indistinguishable from wildtype embryos at 24 hpf though minor motility defects become apparent by approximately 48 hpf. At this point their swimming behavior becomes erratic. Despite initial characterization stating that *jam* homozygotes show weak contraction of the heart (Chen et al., 1996; Granato et al.,

1996; Stainier et al., 1996), I did not observe any cardiac defects in these embryo. With the exception of the slight mobility problems and the lack of a swim bladder, these embryos are otherwise normal (Figure 3.30).

3.13 Mutant muscle structure

The trunk muscle of both *herzschlag* and *still heart* homozygous embryos does not have the striated appearance evident in wildtype animals when observed under DIC (Figure 3.31B, C). Similar to the *unc45b* morphants (Figure 3.19), this tissue becomes vacuole-filled at 5 dpf presumably due to necrosis of the muscle (Figure 3.31F, G). As expected from their mild phenotype, *jam* embryos display normal muscle striation at 48 hpf but this is not the case at 5 dpf (Figure 3.31 D, H). At 5 dpf they appear to lack striation though they do not develop vacuoles unlike *herzschlag* and *still heart* embryos.

When observing slow muscle organization using immunostaining with the F59 and S58 antibodies (explained in section 3.8), *herzschlag* embryos appear to be the most severely affected. At both 24 hpf and 48 hpf, these proteins do not form any organized structure (Figure 3.32). Instead the staining appears punctate possibly indicating aggregation of the myosins that these epitopes recognize.

The F59 and S58 immunostaining pattern in *still heart* homozygotes is similar to that seen in *unc45b* morphants (Figure 3.33). At 24 hpf, neither the F59 nor S58 epitopes are present in any striated pattern; instead, only diffuse staining is observed. By 48 hpf the F59 epitope gains near wildtype organization though the structures appear to be

interrupted by the single nucleus of the slow muscle cell while the S58 staining pattern remains diffuse.

F59 and S58 immunostaining is again indistinguishable from the wildtype staining pattern in *jam* embryos, consistent with their near normal motility (Figure 3.34). This procedure was not performed at 24 hpf as *jam* homozygotes could not be differentiated from their wildtype siblings earlier than 48 hpf.

3.14 Heart structure in mutants

Like *unc45b* morphants, *herzschlag* and *still heart* embryos display numerous cardiac abnormalities (Figure 3.35, Figure 3.36). The hearts fail to undergo correct morphogenesis and do not loop properly. Chamber differentiation remains unchanged in these embryos as evidenced by the unaltered staining pattern of *myh6* and *vmhc*. As in *unc45b* morphants, *myl7* is upregulated in the atrium of these mutants. At the translational level, the atrium myosin heavy chain is localized normally as observed by immunostaining with the S46 antibody. The epitopes corresponding to the F59 and MF20 antibodies, like the *myl7 in situ*, are upregulated in the atrium of the mutant embryos when compared with their wildtype siblings. Again the *jam* mutants do not demonstrate any staining patterns, either by *in situ* hybridization or immunostaining, that differ from those of their wildtype siblings (Figure 3.37).

3.15 Jaw structure in mutants

The cranial structure of both *herzschlag* and *still heart* is severely disrupted (Figure 3.38, Figure 3.39). As is common in embryos lacking circulation, their heads are smaller and underdeveloped (Andrew Waskiewicz, personal communication) making it difficult to tell if all cartilages are even present. On a gross level, one can observe that jaw structure is affected in live embryos (Figure 3.38D, Figure 3.39D). Alcian blue staining demonstrates that the Meckel's and palatoquadrate cartilages are shorter and basihyal appears malformed (Figure 3.38F, Figure 3.39F). The basihyal, ceratohyal, basibranchial and ceratobranchial cartilages are also ventrally displaced (Figure 3.38E, Figure 3.39E) (see Figure 3.26G and H for diagram of cartilages).

There are no obvious defects the cranial region of *jam* embryos however upon examination with Alcian blue staining, it appears that the Meckel's cartilage may be slightly shorter and less rounded than wildtype. When observed from a lateral point of view this cartilage appears to be unaffected (Figure 3.40).

3.16 *unc45b* in mutants

3.16.1 Sequencing of *unc45b* cDNA from *herzschlag* and *still heart*

Due to the similarity between the phenotypes of the *herzschlag* and *still heart* mutants and the *unc45b* morphants, I sequenced the *unc45b* cDNA from homozygous mutants to look for coding changes that may reveal the causative mutation in these strains. I did not uncover any coding changes in the *unc45b* coding region in either

strain. I did not perform this using the *jam* mutants because of the dissimilarity of the mutant and *unc45b* morphants phenotypes and because I was not confident in the identification of the homozygous *jam* embryos.

3.16.2 *In situ* hybridization

Although the sequencing of the *unc45b* coding region from *herzschlag* and *still heart* homozygotes did not reveal any coding changes, the possibility remained that the causative mutations resulting in their respective phenotypes could reside in a non-coding promoter region that could alter the expression pattern of *unc45b* at the transcriptional level. Therefore I performed *in situ* hybridization using a DIG-labeled *unc45b* antisense probe and compared the expression patterns of the homozygotes and their wildtype siblings. I did not observe any downregulation of *unc45b* expression in these mutant strains (Figure 3.41, Figure 3.42). *unc45b* may be slightly upregulated in the somites of the mutants, possibly due to a biological attempt to compensate for the absence of the proteins affected by the mutations present in these strains.

Table 3.1. Exon and intron sizes of *unc45a*

Exon #	Exon Length	Intron #	Intron Length
1	177	1	2376
2	202	2	89
3	176	3	82
4	90	4	2142
5	167	5	1797
6	170	6	3110
7	171	7	97
8	172	8	2909
9	301	9	952
10	95	10	2791
11	142	11	93
12	141	12	2528
13	128	13	266
14	67	14	3810
15	114	15	352
16	116	16	2180
17	118	17	1463
18	156	18	3172
19	407	19	183
20	177		

Genomic organization was determined according to genomic clone XX-95K20 (Accession number AL935028.14) using *unc45a* sequence provided by Dr. Beth Roman. Exon 1 begins with nucleotide 9 of cDNA sequence given in Appendix II.

Table 3.2 Exon and intron sizes of *unc45b*.

Exon #	Exon Length	Intron #	Intron Length
1	107	1	147
2	177	2	79
3	37	3	1027
4	176	4	77
5	90	5	1296
6	168	6	3311
7	165	7	81
8	172	8	2700
9	173	9	1779
10	300	10	1686
11	95	11	1506
12	142	12	99
13	141	13	2006
14	128	14	413
15	67	15	93
16	113	16	123
17	117	17	1830
18	118	18	75
19	156	19	1840
20	1167		

Genomic organization was determined according to genomic clone CH211-20402 (Accession number CR847826.10) using *unc45b* sequence (Accession number AF330001). Exon 1 begins with nucleotide 24 of cDNA sequence given in Appendix IV.

Table 3.3 Effect of morpholinos at various concentrations

Observed phenotypes	Amount of morpholino injected per embryo							
	<i>unc45b</i> MO (1)				<i>unc45b</i> MO (2)			
	0.5 ng	1 ng	5 ng	10 ng	0.5 ng	1 ng	5 ng	10 ng
paralysis at 24 hpf	40.1% (n=142)	90% (n=140)	97.6% (n=124)	95.8% (n=72)	5.3% (n=170)	9.6% (n=125)	86.6% (n=134)	86.7% (n=128)
lack of circulation at 48 hpf	48.8% (n=123)	85.1% (n=101)	96.1% (n=76)	97.4% (n=38)	1.6% (n=129)	15.4% (n=65)	92.8% (n=125)	96.2% (n=105)
ventricular dysfunction at 48 hpf	10.6% (n=123)	75.2% (n=101)	96.1% (n=76)	92.1% (n=38)	0% (n=129)	1.5% (n=65)	60.8% (n=125)	72.4% (n=105)

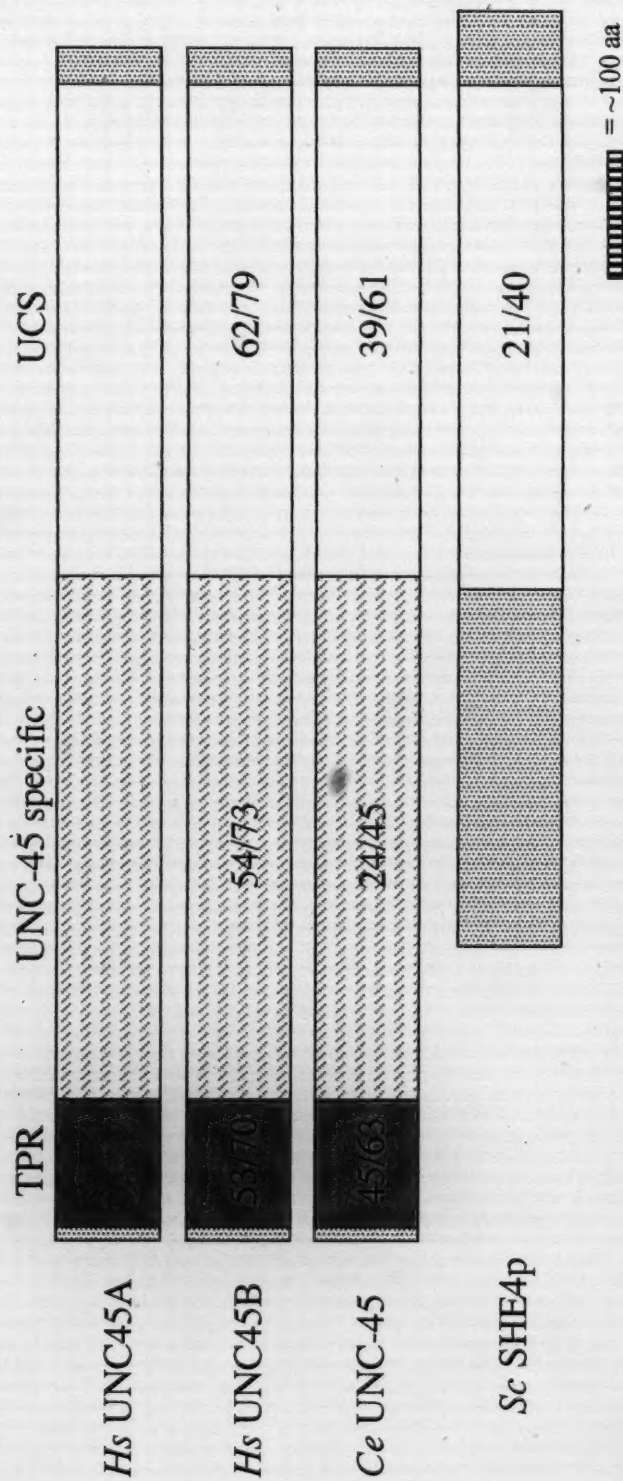


Figure 3.1 Comparison of human UNC45A with other UCS proteins
Homo sapiens UNC45A is compared to *Homo sapiens* UNC45B (accession number AA013384.1), *Caenorhabditis elegans* UNC-45 (accession number AAD01976) and *Saccharomyces cerevisiae* SHE4p (accession number CAA63795.1). X/Y represents the percentage identity and similarity where X is the percent identity and Y is the percent similarity between the indicated protein and *Homo sapiens* UNC45A (accession number AAH37992.1).

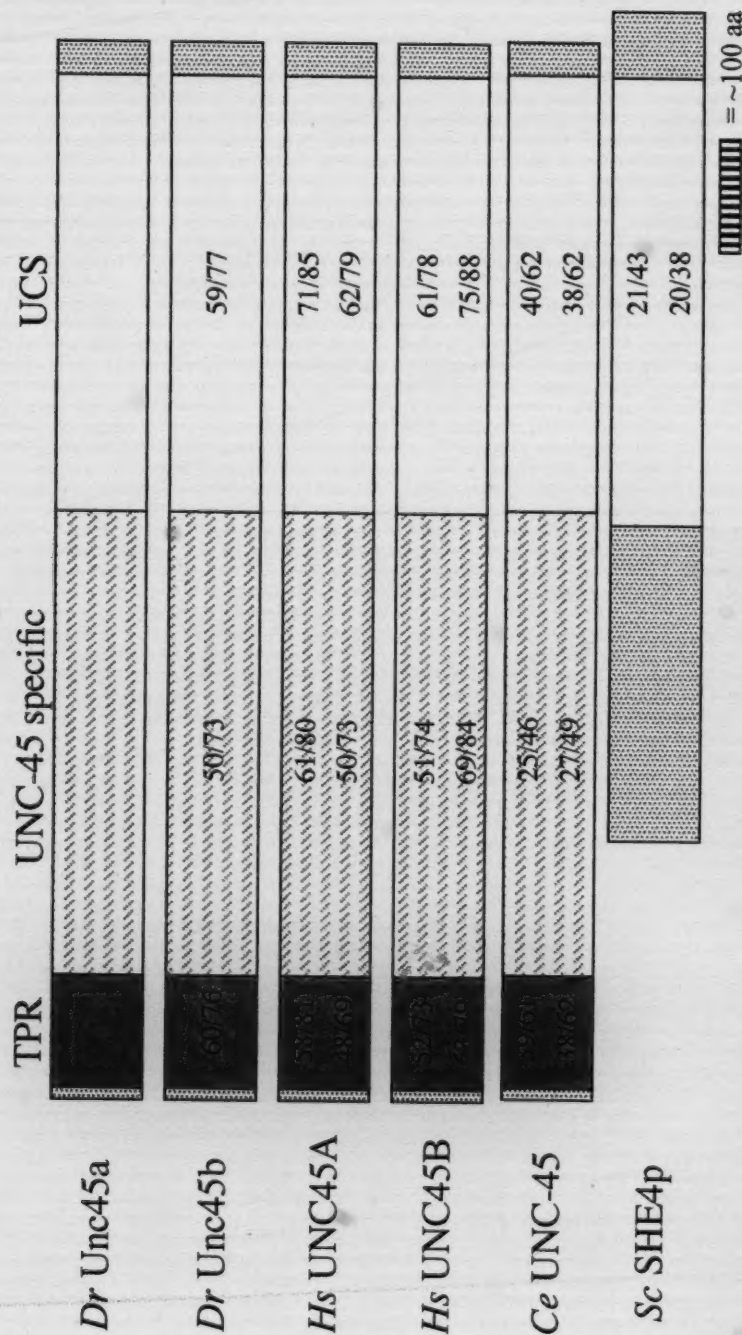


Figure 3.2 Comparison of zebrafish Unc45 proteins with other UCS proteins
Danio rerio Unc45a (Beth Roman, personal communication) and *Danio rerio* Unc45b (accession number AAL57031) are compared to *Homo sapiens* UNC45A (accession number AAH37992.1), *Homo sapiens* UNC45B (accession number AAO13384.1), *Caenorhabditis elegans* UNC-45 (accession number AAD01976) and *Saccharomyces cerevisiae* SHE4p (accession number CAA63795.1). X/Y represents the percentage identity and similarity where X is the percent identity and Y is the percent similarity between the indicated protein and *Danio rerio* Unc45a (shown as top numbers where applicable) or *Danio rerio* Unc45b (shown as bottom numbers where applicable).

Unc45a	-----K6QSSMREECNHFAGDVQOALCYTKAKKISDCPSE--SAVLYRNRSA CYLKEDETTKAEEDATKSLDVPDQDIKARFRAC	84
Unc45b	NTMGHICDSVQLKEGNNHFQAGEIDQALDCTYKAIKICKEDKAKAVIYRHSACFLKFNENSGNSATKAIDVDAADIKALYRRC	90
Unc45a	ELQKLGRLDQAFDVOKCAOLEPKNKMEODLRLGLQIQOKAQLSTDSRVQOMFELDDSSAPADCKAAQNLAVLSREDAGAFQ	174
Unc45b	AFEXKLGKIDMAFDVQRCATTIEPKNKMELEQLRLGLGASIQQLKLTSTDSRVQNHFIPLSDPEDEKEMKKAANNLIVLAREDAGER	180
Unc45a	ERNQVGLQNLLESKQBELILSALRTIVGLSTGHQHRMAIVNELGHERLCGVNGCSASVHLSACHLTQVMPFALTTEGKMLIFKDBE	264
Unc45b	FQNGVPLLMQLITGCKPEGLAAARTLSCACTGHRARATALHISVGLISKLCSIMAVDNEELIALATANTFCVNDLSLG-GDARNYCKEE	269
Unc45a	ALLPSPRELSMRHLLDMPASSVSCAGRDSAINLWKQVPEKSVKNDNSLSLAVIDQGLAKILFPACTVAEYENGFPLENTNMSC	354
Unc45b	ALVPLSSKDLADILALALEMIAKRVSGHGRDQALNTAKRVPRQNKSTDNSKCLFTIDGLKKILKVCQVDPDLPDQLEPENTQOLIA	359
Unc45a	SVLLAKLYDDLSKQERENESKLCCEYVQHFSSSMERLRAIQTVSVLQGSVDGVNVTLELSGLMDSVISLCASEDIAQGVAVEAL	444
Unc45b	SVLLSKLYDDLRQFPERDQFNDICDDYIKSEFPNDMDENHAIINTLSCHLQGFPLDIGNYLAGRQGVMDAVLCCGSEREVDTLVAVEAL	449
Unc45a	THAAQFAKASFTIANGVALIKETKESQNDRIHVHALVGLCKIGSAGGQDFSMKQFAEGSTPKLAKQCRKMLCNSLPPAGRWAIQGL	534
Unc45b	IHAGTKSKASFFIENGVSILKEVKKTKQNELIIIRALVGLCKIGSAGGQDYSMRQFAEGSTPKLAKQCRKMLCNSLPPAGRWAIQGL	539
Unc45a	AYLRLDADVKEDAVEDAKALQMPFLKSDQKTVLFAVCTLVNCTNSYDVKEPDPQAVGLAKYAKQHVPEBPDKQGFVQVVKLLE	624
Unc45b	AYLRLDADVKEDYAEDEFAKRAMPELQKSDKTLILYAVCTLVNCTNSYDKEEIIPEMLQAKFSQHVPEBPDKQGFVQVVKLLE	629
Unc45a	AGVTSALVCMVKQESPAKTEACRECHARTIALVERQEDRGLVFAQCGGKALLPLVSESTDRCCKIKAAQALAKITITSNPEIAFPGERVY	714
Unc45b	AGVTSALAVVVEADNSILTDQTEFLARVFTALTEDVDRGLIIVAQCGGKALLPLALEGTDKCKIKASALAKITAAVSNPEIAFPGERIY	719
Unc45a	EVRPLVSLALDCSMLQNFELALTNLAGISERLRKIIKEKAVPNIECYMFEEDHVRMASTECMCNIALSTEVQILYATESDLK	804
Unc45b	EVRPLVSLIGTDQGHENFEALGLTNLAGLNKLRKILKEKALPLFETNPFEDHEQIRQAATECMCNLVCCKEVQDRLEDDNDKLR	809
Unc45a	LLVTVSGEDDERKKAAGTLLAVTQEMPELCTIPDTQSHLEILLQALLQESQDLRHRGVVIVNIMQADKSLAKLINESPALTISV	894
Unc45b	LLVTLCEDEDEKQRAAGALNLTIAKKKAVENTKVTSQMLEILQRCIDNNPFOHRGLVTVFVMLDADQOLNKLIVESDMEITITY	899
Unc45a	PLTQDD--FKQASVRRKQRCQTDLALEYGLTIRSNESGVNGNSI	935
Unc45b	VALLEDNEKQNAIDARACLSMNGGLTFPSN-----	934

Figure 3.3 Protein alignment of the zebrafish Unc45a and Unc45b homologues

Identical amino acids are shaded in black. Similar amino acids are shaded in grey. Broken lines indicate gaps in the alignment.

Drerio	mta	60
Hsapiens	---	55
Celegans	---	59
Scerevisiae	-----mpicckndpidsstidelcaa[dktk]ka-p-----	31
Drerio	---	120
Hsapiens	---	115
Celegans	---	119
Scerevisiae	-----dvqkyndaintifqlrqksesgkmpadltensea[kdrck]---	70
Drerio	---	178
Hsapiens	---	173
Celegans	---	179
Scerevisiae	ieci[tray]chsearvhlslkligndipfaln-----	102
Drerio	---	230
Hsapiens	---	225
Celegans	---	239
Scerevisiae	-----lfe[il]ers-----sihvfv[cfsn]--[d]at[i]a[ll]ne[il]q-----	133
Drerio	---	288
Hsapiens	---	284
Celegans	---	299
Scerevisiae	---[l]rihyge[thvtylls][il]q[lnkfkynfkevrlvk][il]lrisedevksam[il]lifa	190
Drerio	---	348
Hsapiens	---	344
Celegans	---	358
Scerevisiae	cl[gs]-----f[k]df[kavvdfmsslivea][dv]ndp[isil]vkt[le]alyps	237
Drerio	---	403
Hsapiens	---	399
Celegans	---	418
Scerevisiae	[lt]t-----lcsel[fltk]slk[il]kfkks-v[es]qdl[et]kellr-----	275
Drerio	---	463
Hsapiens	---	459
Celegans	---	478
Scerevisiae	-----[l]lss[cid]etmrtyit[ny]q[ll]e[sl]nvedvq	308
Drerio	---	523
Hsapiens	---	519
Celegans	---	537
Scerevisiae	iy[al]v[vt]k[efk]l[tc]n[k]q[se]l[fin]a[is]r[rim]pkienv[...]	357
Drerio	---	580
Hsapiens	---	578
Celegans	---	595
Scerevisiae	---	413

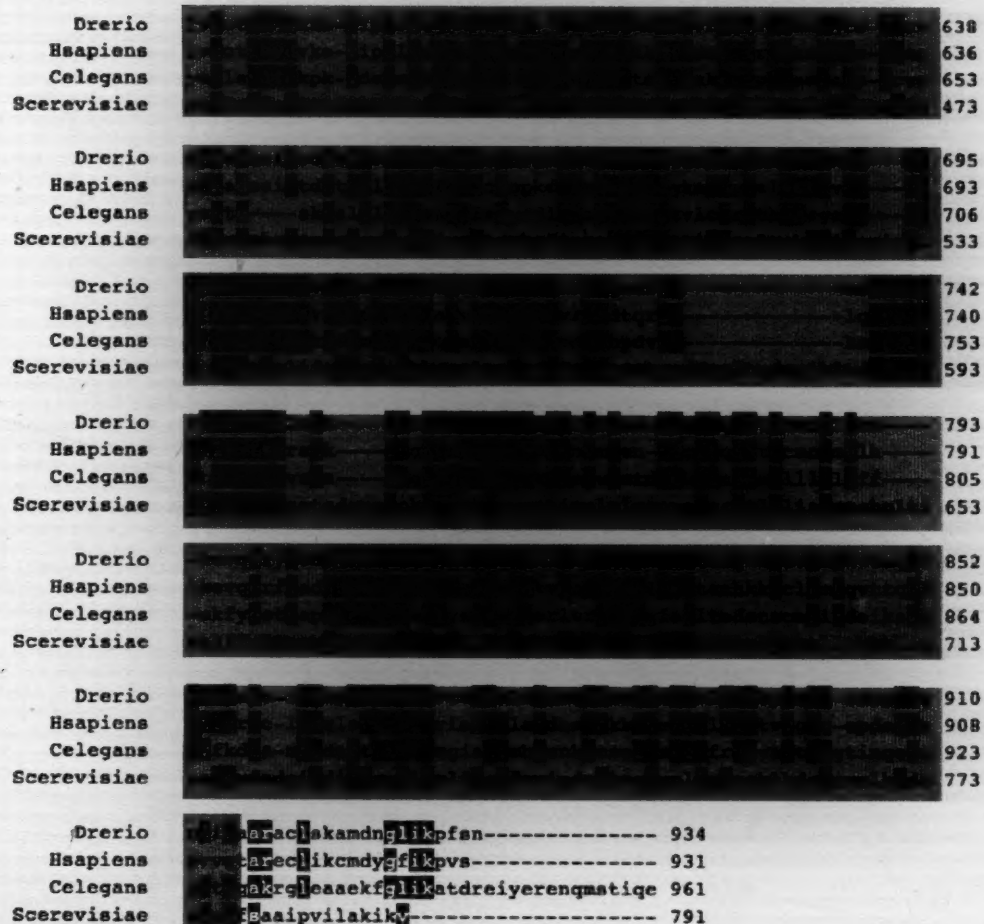


Figure 3.4 Protein alignment of zebrafish Unc45b and its human, *C. elegans* and yeast homologues

Zebrafish Unc45b (Drerio), Human UNC45B (Hsapiens), *C. elegans* UNC-45 (Celegans), and *S. cerevisiae* She4p (Scerevisiae) are aligned. Identical amino acids are shaded in black. Similar amino acids are shaded in grey. Broken lines indicate gaps in the alignment. TPR domain (pink), unique central domain (purple) and UCS domain (blue) are shaded to indicate approximate positions.

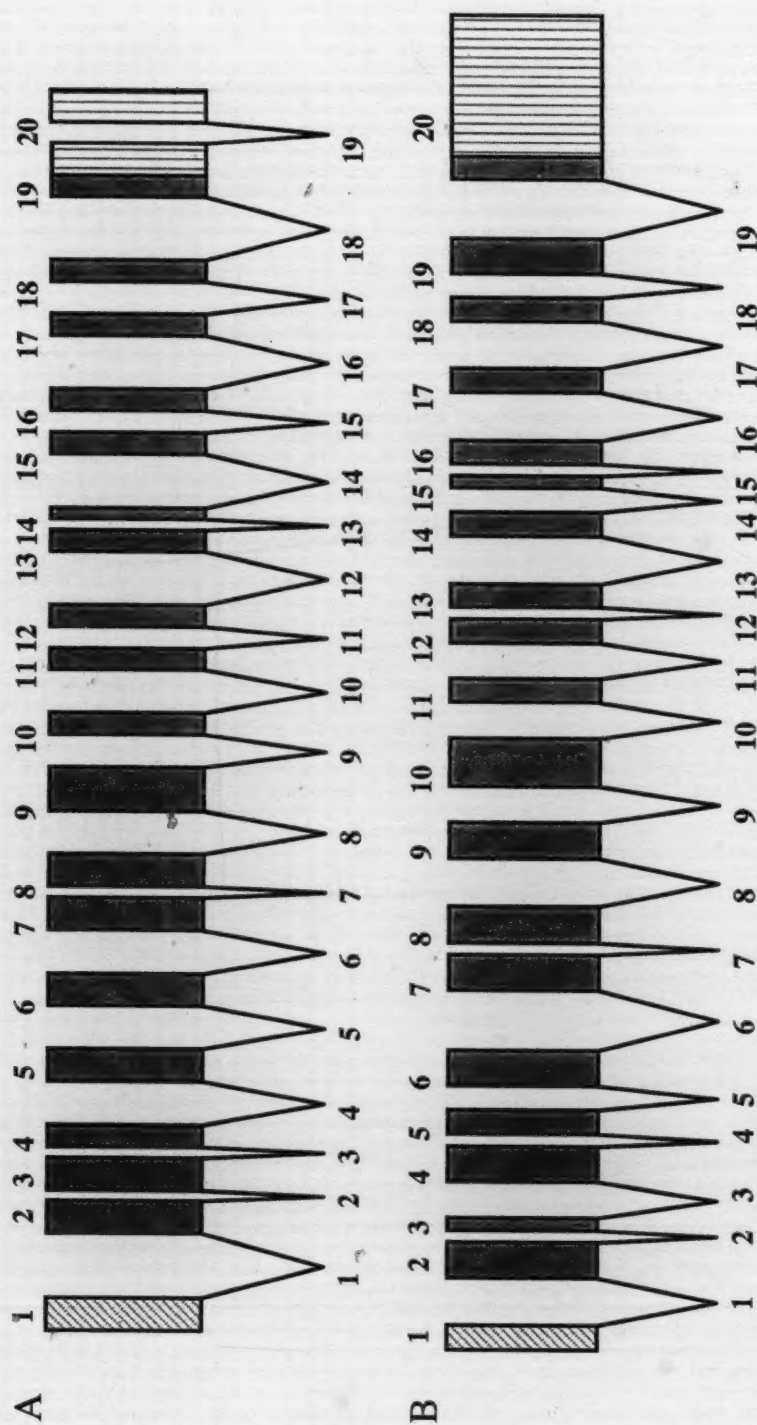


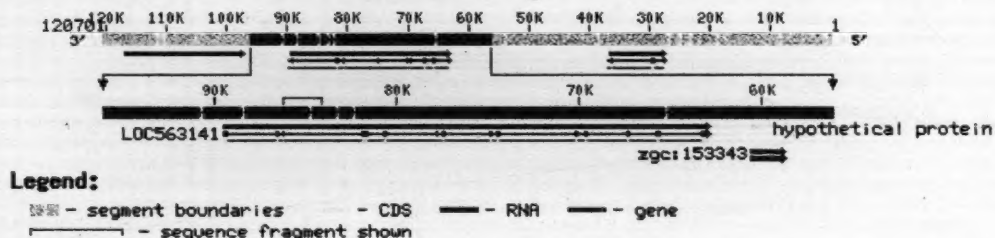
Figure 3.5 Genomic organization of the *unc45a* and *unc45b* genes

(A) Genomic organization of the *unc45a* gene. Total genomic size is 33673 bp. For intron and exon sizes see Table 3.1.

(B) Genomic organization of the *unc45b* gene. Total genomic size is 25302 bp. For intron and exon sizes see Table 3.2.

Exons are shown as upper grey boxes and introns shown as lower pointed lines (not to scale). Diagonal striped boxes indicate 5' UTR. Vertical striped boxes indicate 3' UTR. Solid grey boxes indicate coding region.

A



B

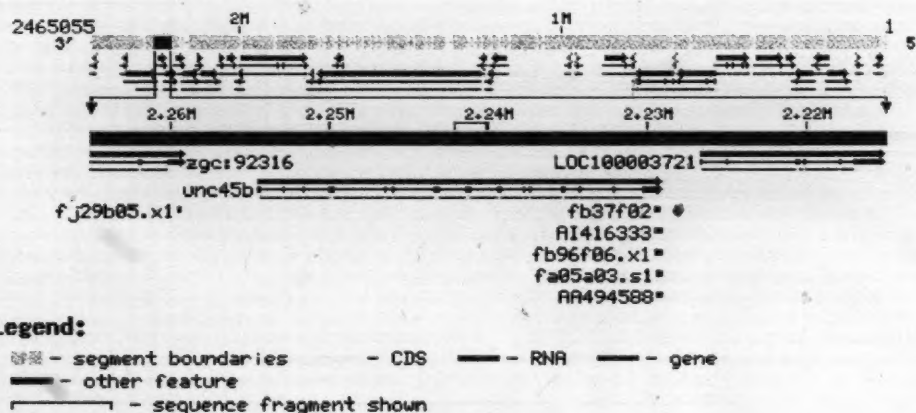


Figure 3.6 Genomic regions containing the *unc45a* and *unc45b* genes
 (A) Genomic region (accession number NW_001516887 containing the *unc45a* gene. In this view *unc45a* is called LOC563141. (B) Genomic region (accession number NW_001513913) containing the *unc45b* gene. Each of these views are the reverse complement of the actual strand as these genes lie on the minus strand. Gray regions indicate genes. Dark blue and pink regions indicate exon locations. Views are adapted from <http://www.ncbi.nlm.nih.gov>

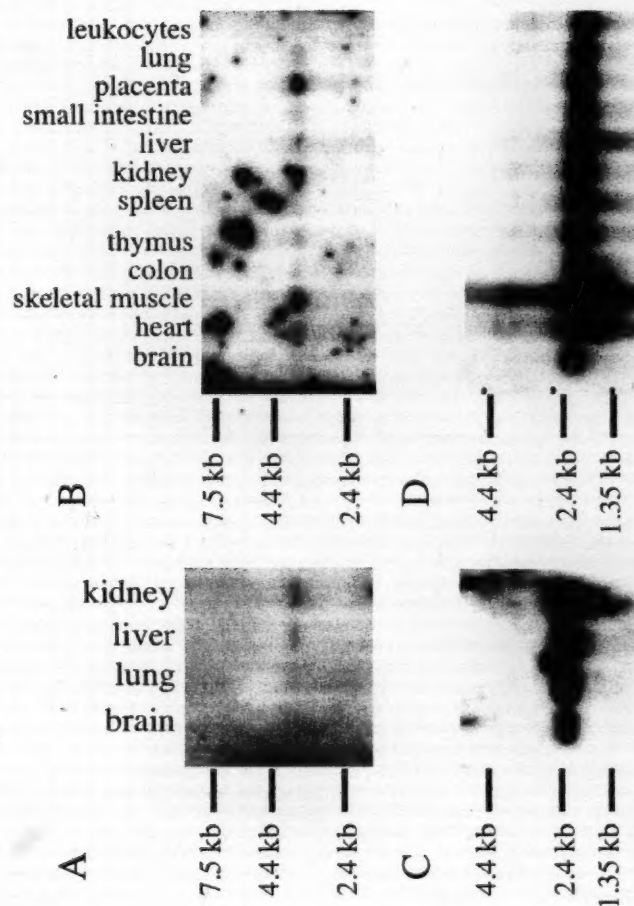


Figure 3.7 Northern analysis of human *UNC45A* (A,C) Fetal human multiple tissue northern blot probed with radiolabeled (A) human *UNC45A* or (C) β actin. (B,D) Adult human multiple tissue northern blot probed with radiolabeled (B) human *UNC45A* or (D) β actin. The β actin probe was used as a control to ensure that gels were loaded evenly during preparation.

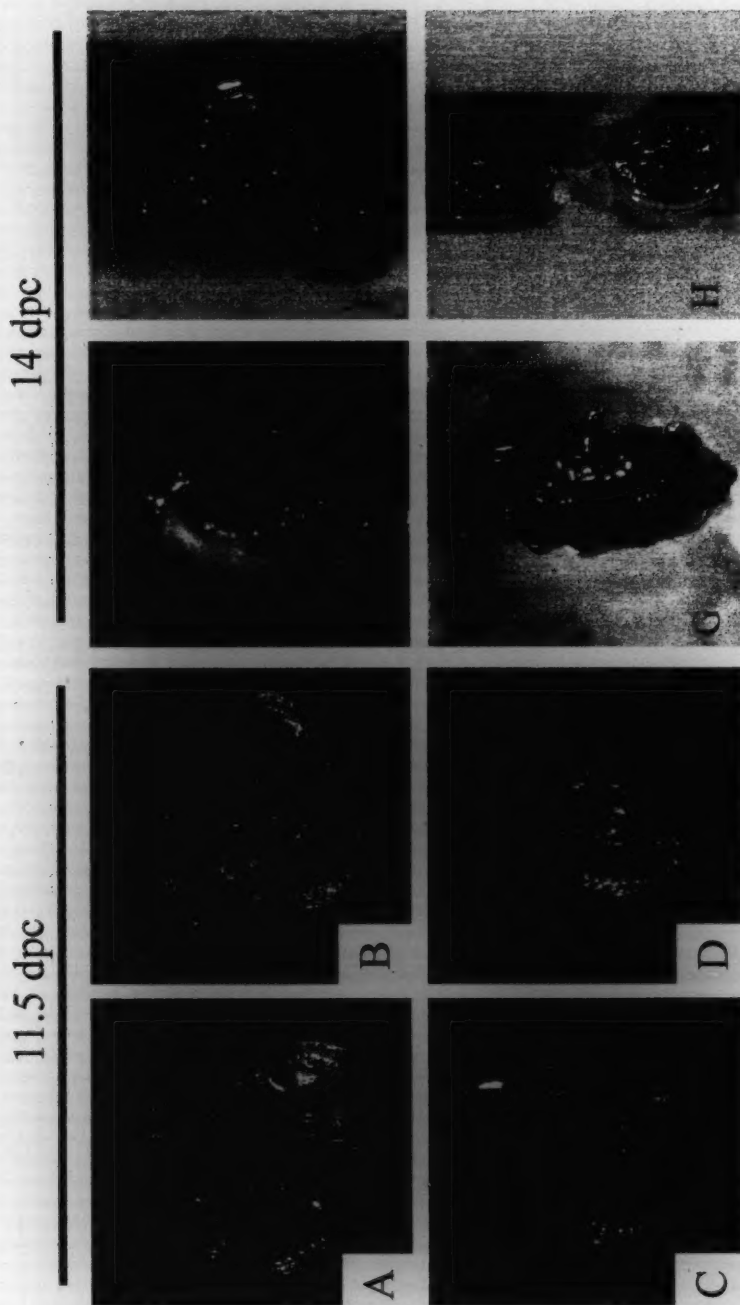


Figure 3.8 *In situ* hybridization of mouse embryos (A,B,E,F) Lateral views showing expression of *Unc45a* in (A,B) 11.5 dpc and (E,F) 14 dpc mouse embryos. (C) Lateral views showing expression of *Pax1* in the somites and limb bud at 11.5 dpc (positive control). (D) Negative control embryo at 11.5 dpc (no probe). (G) Dorsal view showing expression of *Ipw* brain and lateral midline at 14 dpc (positive control). (H) Negative control embryo at 14 dpc (no probe). Please note, embryos in (D) and (H) were broken during *in situ* hybridization procedure.



Figure 3.9 Expression pattern of *unc45a*. *In situ* hybridization with DIG-labelled *unc45a* probe. (A) Dorsal view with anterior to the left of a 48 hpf embryo. Expression appears to be ubiquitous with enrichment in the vascular system. (B) Lateral view with anterior to the right of a 26 hpf embryo.

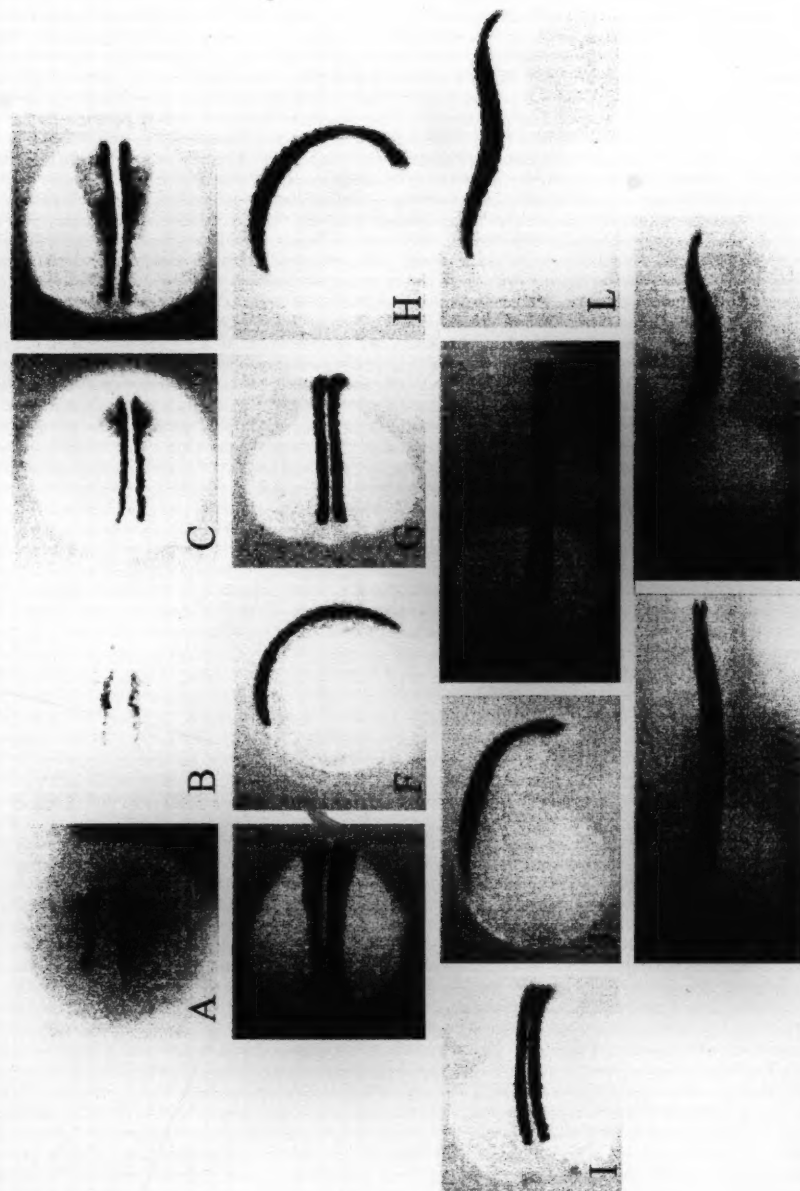


Figure 3.10 Expression pattern of *unc45b* in striated muscle of the trunk
In situ hybridization with DIG-labelled *unc45b* probe. Dorsal view with anterior to left of (A) 9 hpf, (B) 10 hpf, (C) 12 hpf, (D) 14 hpf, (E) 16 hpf, (F) 16 hpf, (G) 18 hpf, (H) 18 hpf, (I) 20 hpf, (J) 20 hpf, (K) 22 hpf, (L) 22 hpf, (M) 24 hpf and (N) 24 hpf embryos. Lateral view with anterior to left of (F) 16 hpf, (H) 18 hpf, (J) 20 hpf, (L) 22 hpf and (N) 24 hpf embryos.

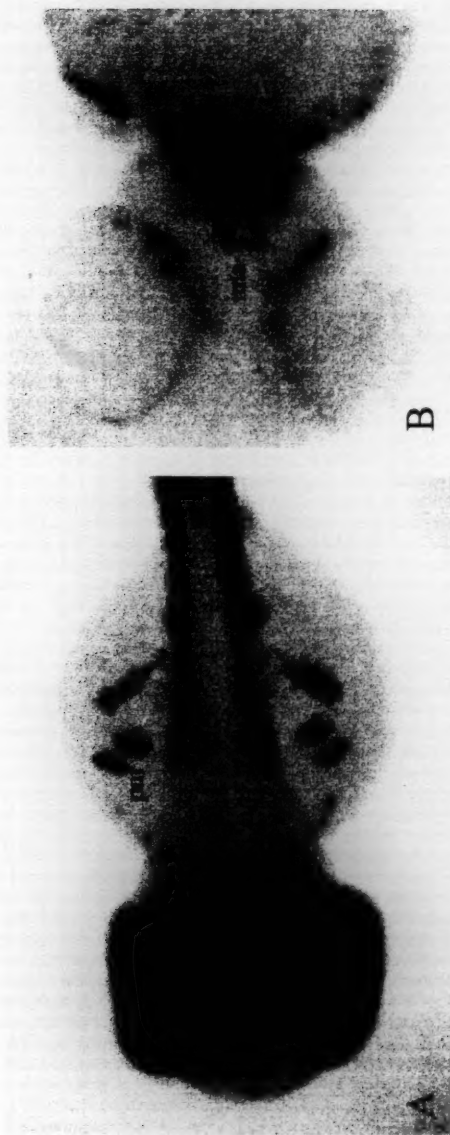


Figure 3.11 *unc45b* expression in other striated muscle
In situ hybridization with DIG-labelled *unc45b* probe. (A) Dorsal view with anterior
to the left of a 48 hpf embryo showing *unc45b* expression in pectoral fin muscle. (B)
Ventral view with anterior to the left of a 3 dpf embryo showing *unc45b* expression in
cranial muscles. hh, hyohyoideus; ih, interhyoideus; ima, intermandibularis anterior;
imp, intermandibularis posterior; ir, inferior rectus; pm, pectoral fin muscle; sh,
sternohyoideus.

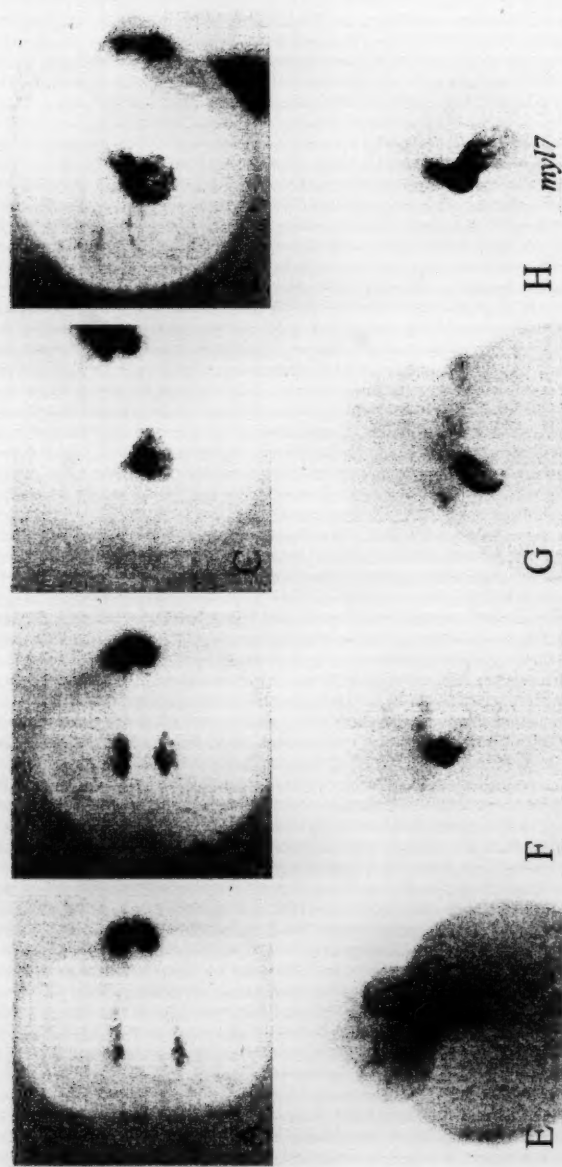


Figure 3.12 Expression pattern of *unc45b* in cardiac mesoderm (A-G) Expression of *unc45b* (determined by *in situ* hybridization with DIG-labelled *unc45b* probe) in the cardiac mesoderm of wildtype embryos. (A-D) Dorsal view with anterior to left of (A) 18 hpf, (B) 20 hpf, (C) 22 hpf, and (D) 24 hpf embryos. (E-H) Ventral view with anterior to top of (E) 36 hpf, (F) 42 hpf and (G) 44 hpf embryos and (H) 44 hpf embryo hybridized with DIG-labelled *myl7* probe for reference.

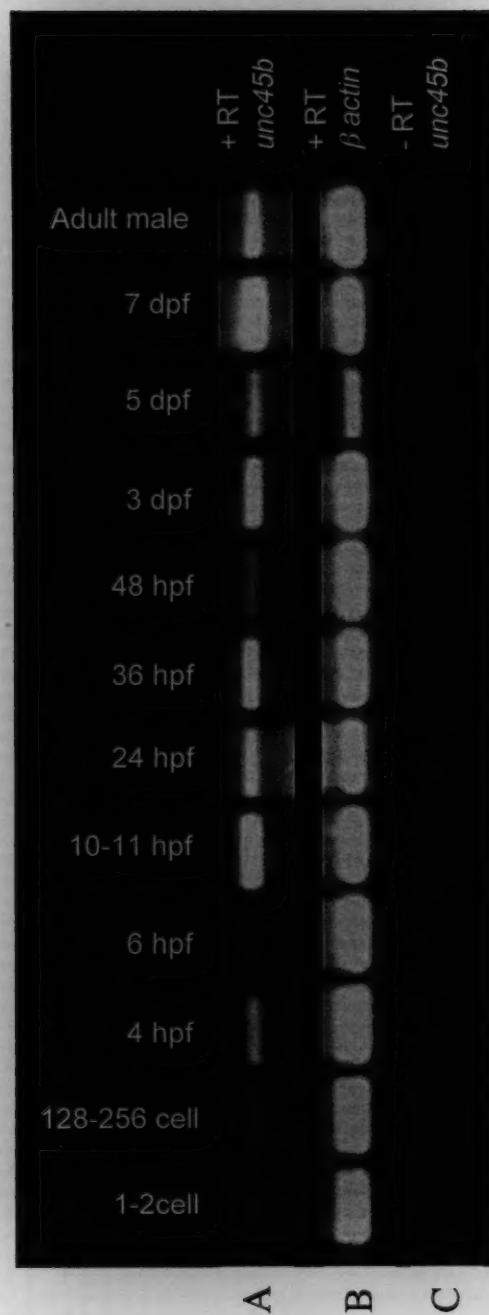


Figure 3.13 RT-PCR to determine expression profile of *unc45b* at various developmental stages (A) RT-PCR using primer specific to *unc45b*. (B) RT-PCR using primers specific to β -actin (positive control). (C) PCR (no reverse transcriptase) using primers specific to *unc45b* (negative control). cDNA was prepared from stages indicated. *Unc45b* transcript is present at all stages tested.

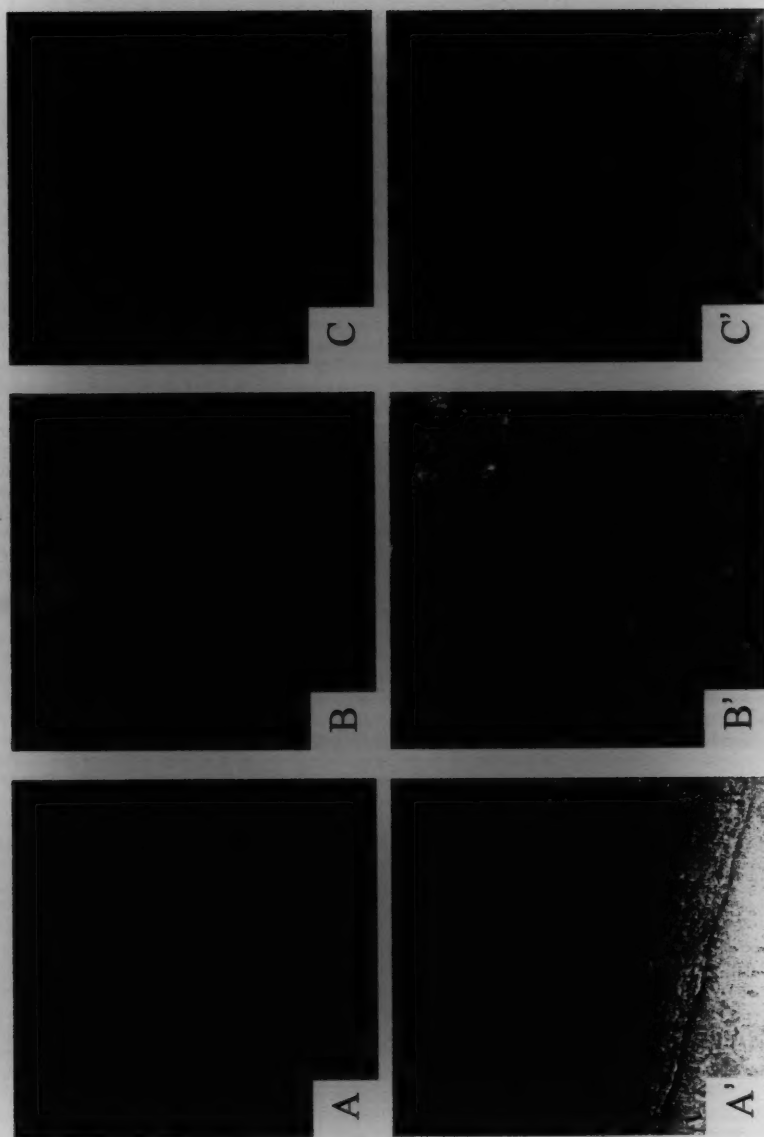


Figure 3.14 Localization of Myc:Unc45b within muscle cells. Immunostaining with anti-Myc antibody of 48 hpf embryo expressing Myc::Unc45b in trunk muscle cells at (A) 63x and (B,C) 63x with 4x optical zoom. (A',B',C') are overlays of fluorescence and DIC images of (A,B,C) respectively.

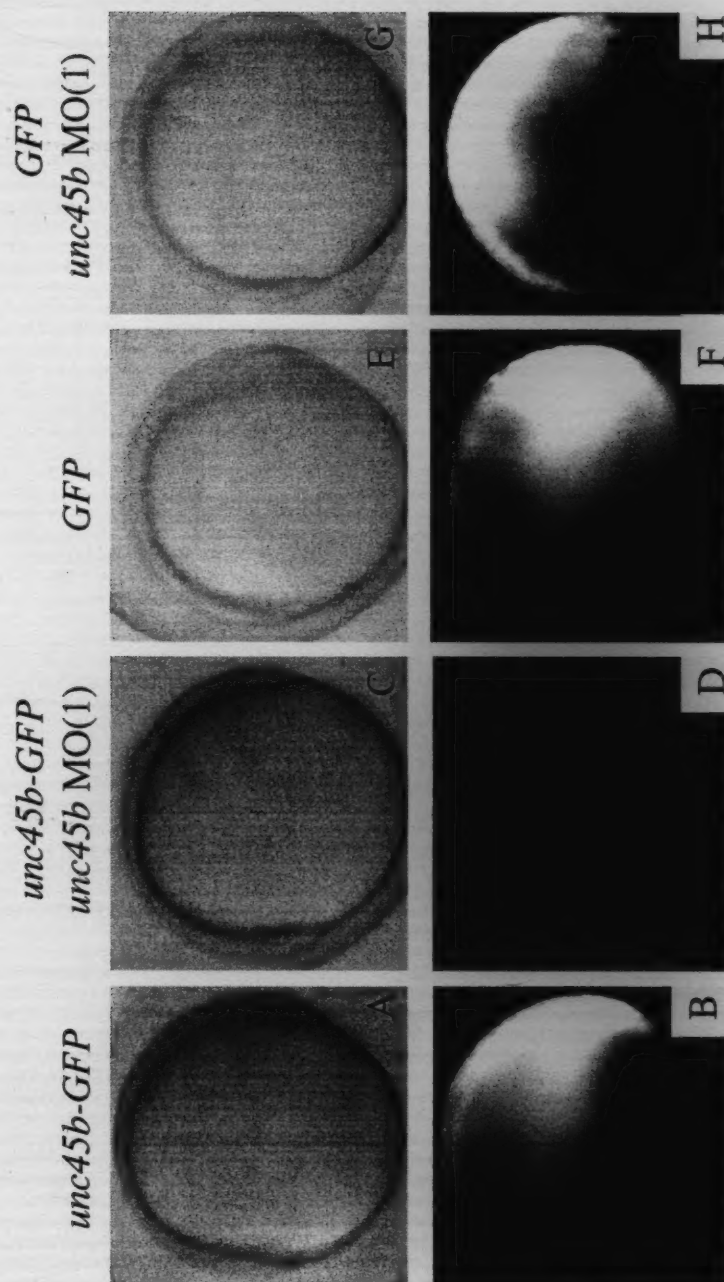


Figure 3.15 Specificity of *unc-45b* MO (1)
 (A,C,E,G) Live and (B,D,F,H) green fluorescent images of approximately 75% epiboly embryos injected with:
 (A,B) 0.2 ng *unc45b::GFP* RNA, (C,D) 0.2 ng *unc45b::GFP* RNA + 2 ng *unc45b* MO (1), (E,F) 0.2 ng *GFP*
 RNA, (G,H) 0.2 ng *GFP* RNA + 2 ng *unc45b* MO (1). GFP expression is completely abolished in embryos
 injected with *unc45b::GFP* RNA and *unc45b* MO (1).

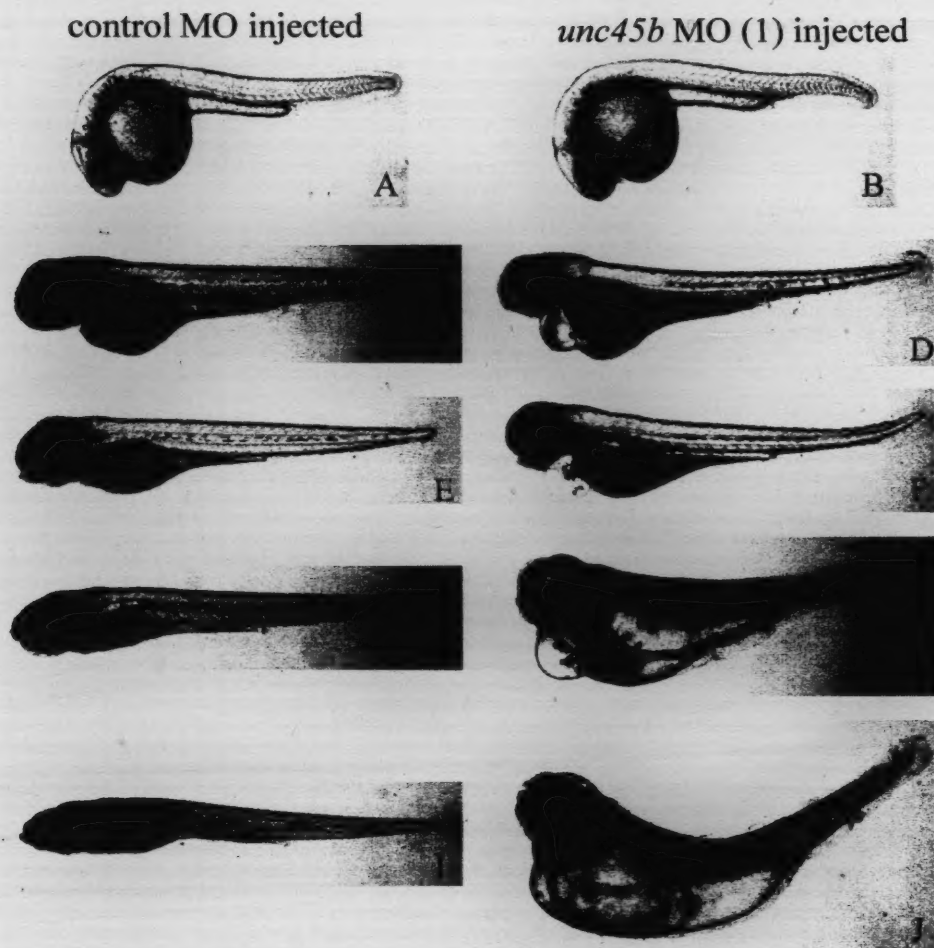


Figure 3.16 Comparison of control MO-injected and *unc45b* MO (1)-injected animals

Photographs of living larva injected with (A,C,E,G,I) 5 ng control MO or (B,D,F,H,J) *unc45b* MO (1) at (A,B) 24 hpf, (C,D) 48 hpf, (E,F) 3 dpf, (G,H) 5 dpf and (I,J) 7dpf. Embryos injected with *unc45b* MO (1) display cardiac and yolk sac edema.

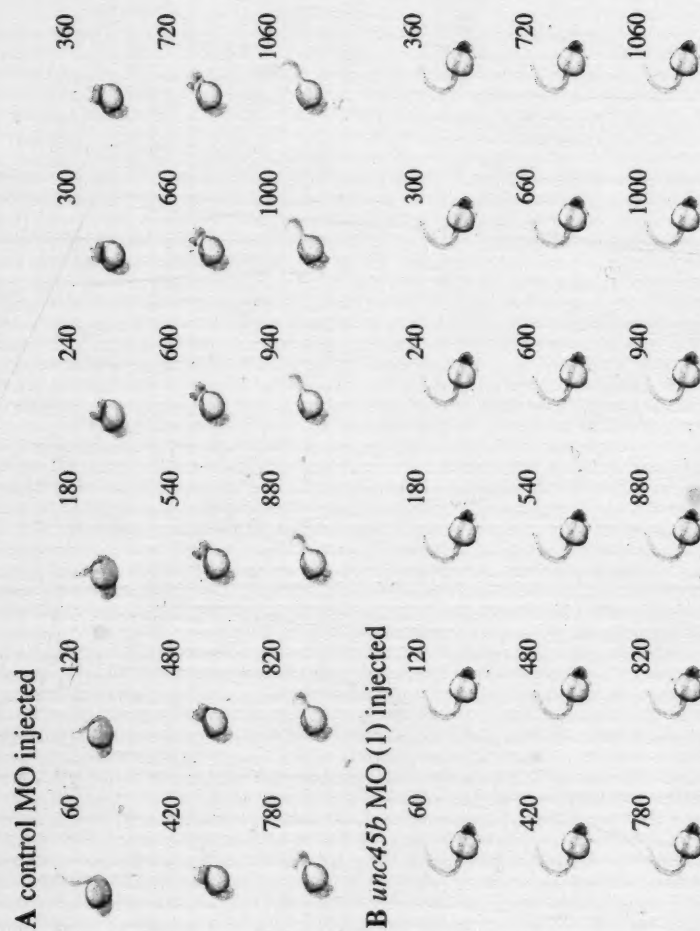


Figure 3.17 Motility of 24 hpf morpholino-injected embryos
Video microscopy of (A) control MO-injected and (B) *unc45b* MO (1)-injected embryos at 24 hpf. Still frames taken at 60 ms intervals are shown. Similar results were observed when embryos were touched with the tip of a pair of forceps (data not shown). Video was collected with a standard digital videocamera at a rate of 30 frames per second.

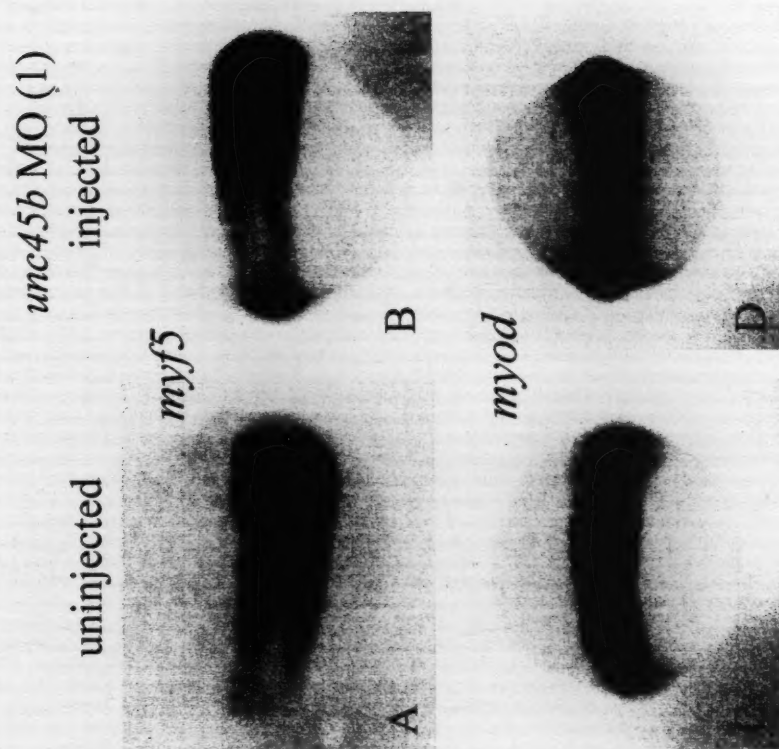


Figure 3.18 Myogenic determination is not altered in morphants. Dorsal views with anterior to the left of approximately 13 hpf embryos. (A, B) Expression of *myf5* in (A) uninjected and (B) *unc45b* MO (1)-injected embryos. (C, D) Expression of *myod* in (C) uninjected and (D) *unc45b* MO (1)-injected embryos.

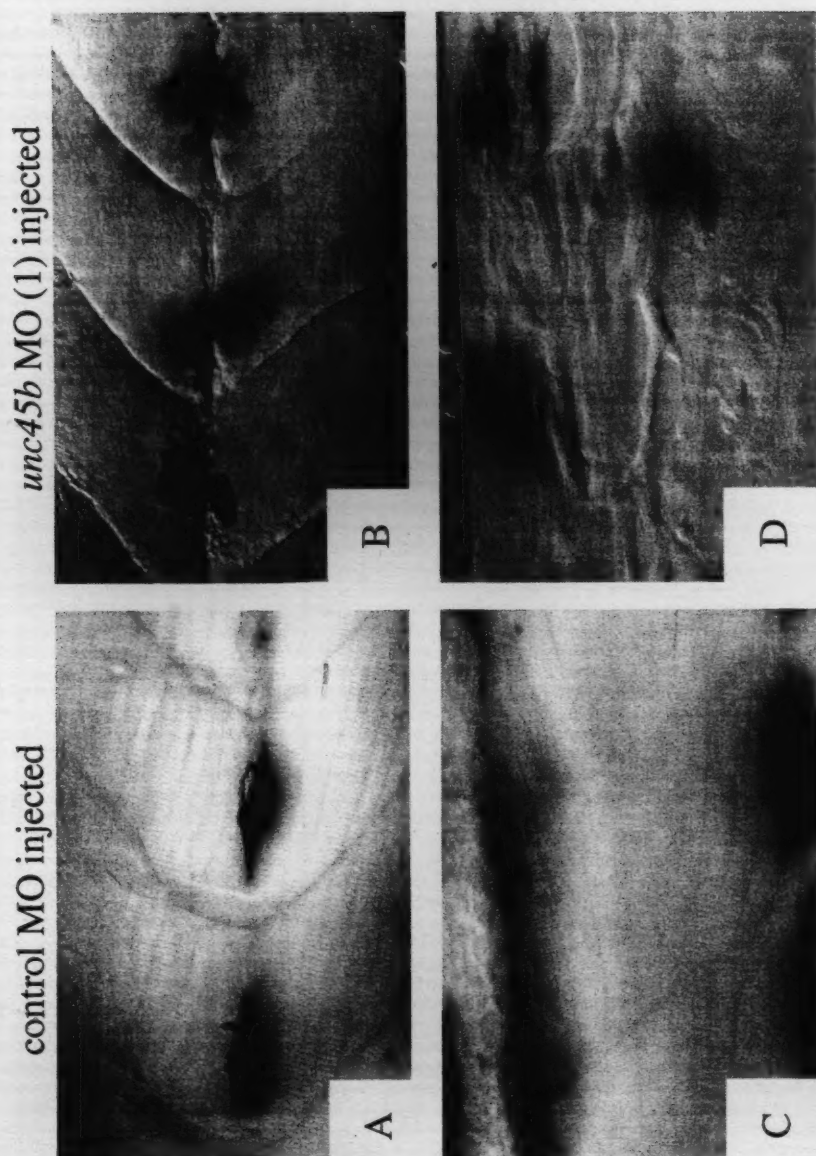


Figure 3.19 DIC photography of trunk muscle
 DIC photographs of lateral views, with anterior to the left, of the trunk living embryos injected with
 (A,C) control MO or (B,D) *unc45b* MO (1) at (A,B) 48hpf and (C,D) 5 dpf. *unc45b* MO (1)-injected
 embryos lack muscle striation and later develop vacuoles in the tissue.

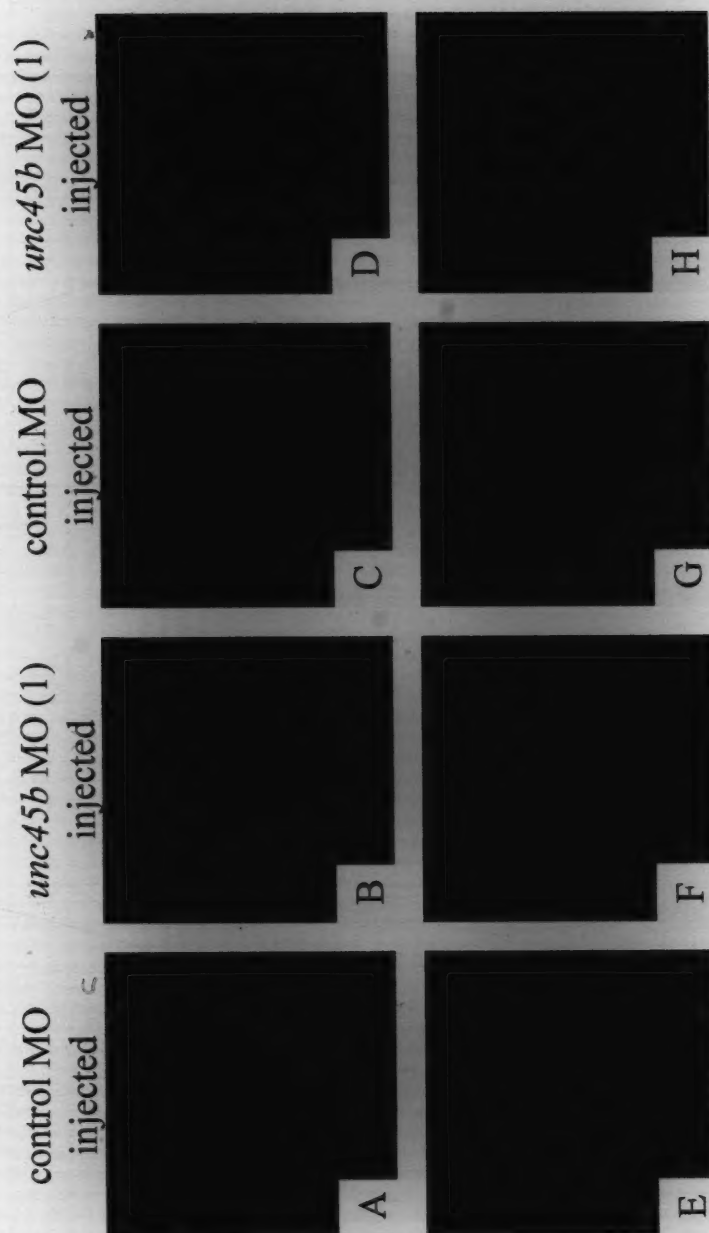


Figure 3.20 Actin filaments at 24 hpf and 48 hpf
Fluorescent phalloidin staining of (A,C,E,G) control MO-injected and (B,D,F,H) *unc45b* MO (1)-
injected embryos at (A,B,E,F) 24 hpf and (C,D,G,H) 48 hpf at 63x magnification. (E, F, G, H) Single
plane of focus and 4 x magnification of (A, B, C, D) respectively.

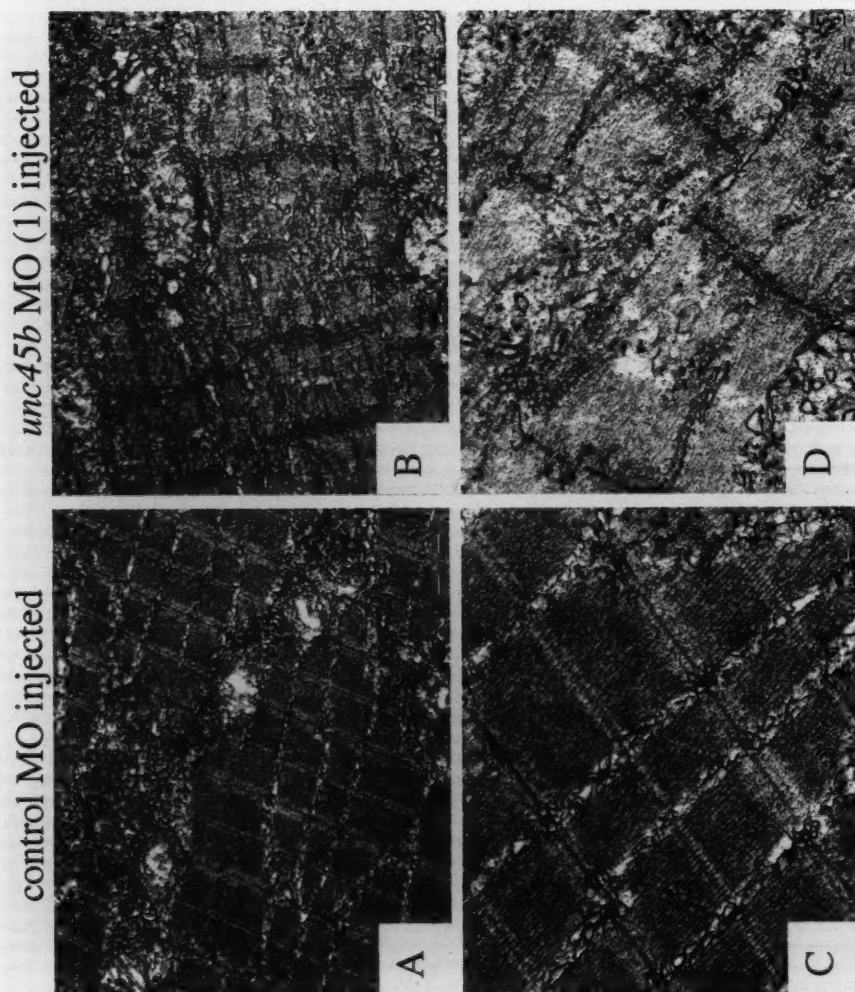


Figure 3.21 Ultrastructure of trunk muscle at 48 hpf
Transmission electron microscopy photographs of sections of trunk muscle taken from 48 hpf zebrafish injected with (A,C) control MO or (B,D) *unc45b* MO (1). Photographs were taken at (A,B) 8900 x or (C,D) 22000 x magnification.

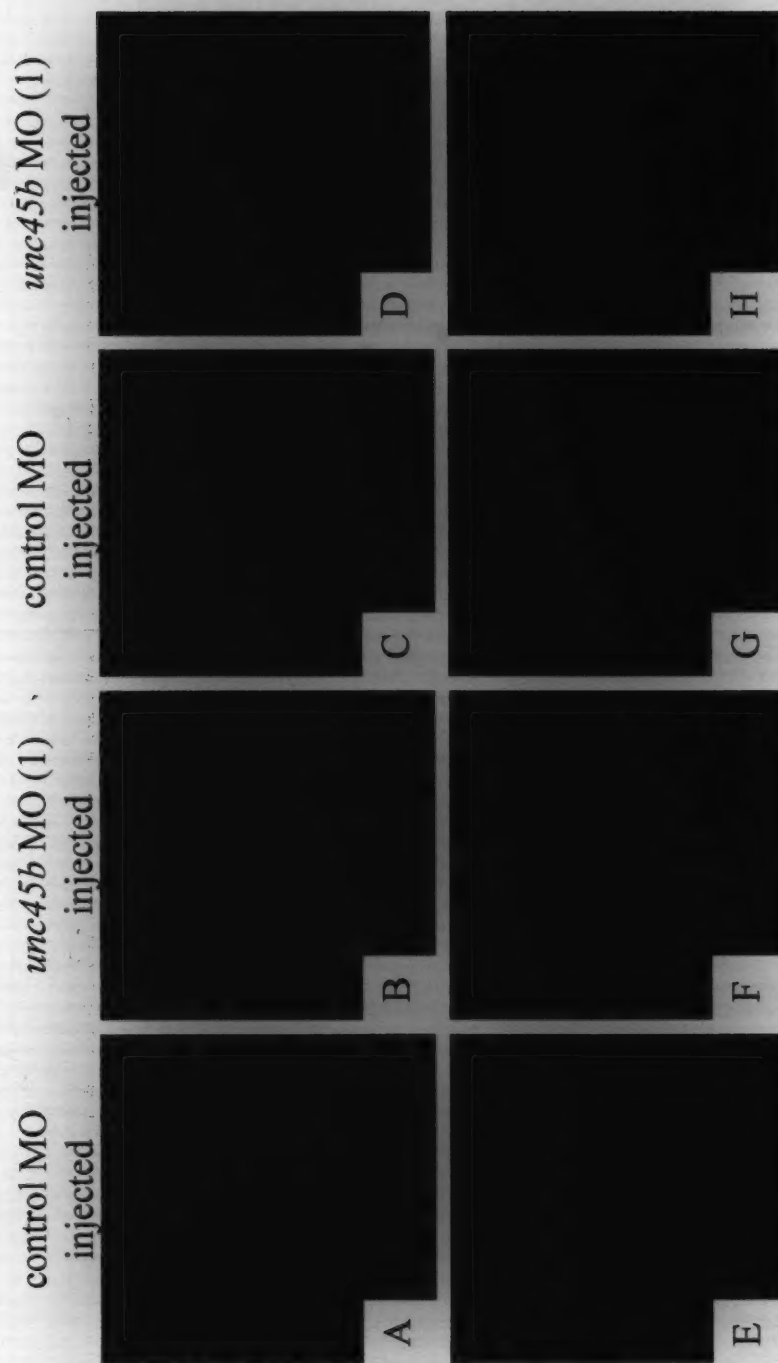


Figure 3.22 Immunostaining with anti-myosin antibodies (A-D) 24 hpf embryos and (E-H) 48 hpf embryos with anterior to the left. (A,B,E,F) F59 staining of the trunk muscle in (A,E) control MO-injected and (B,F) *unc45b* MO (1)-injected embryos. (C,D,G,H) S58 staining of the trunk muscle in (C,G) control MO-injected and (D,H) *unc45b* MO (1)-injected embryos. Photographs were taken at (A-D) 63 x magnification and (E-H) 63x magnification with 4 x optical zoom.

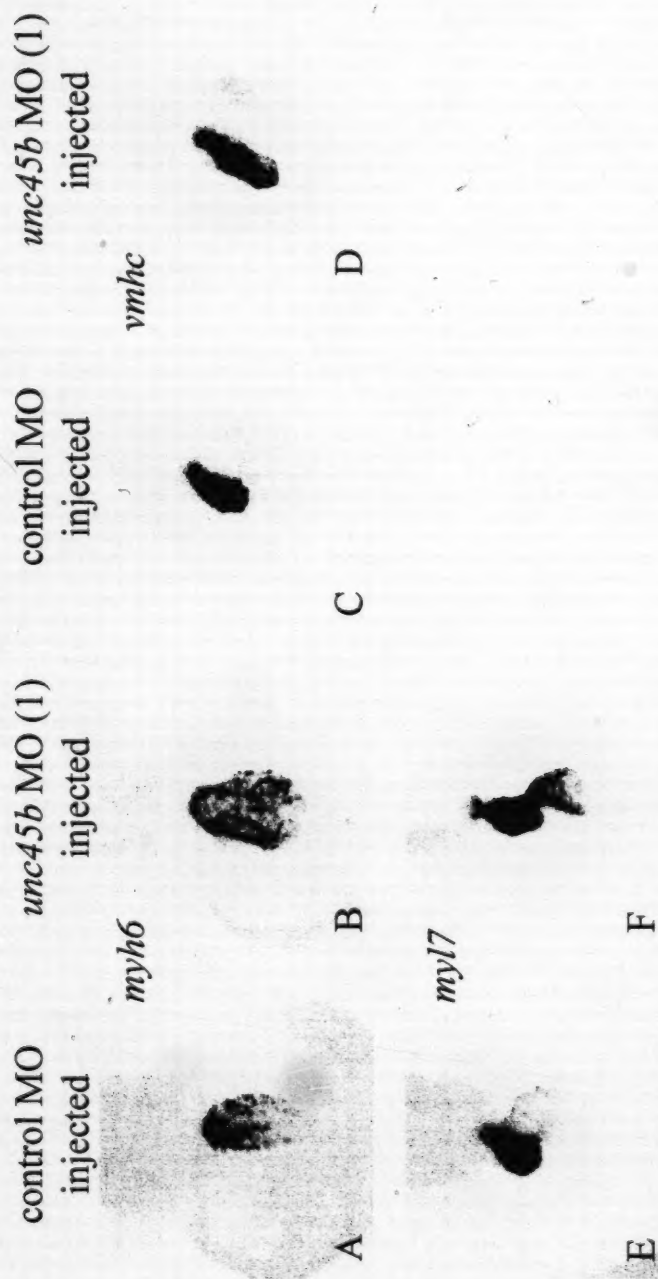


Figure 3.23 Expression pattern of cardiac markers
Ventral views of 48 hpf embryos with anterior to the top. (A, B) Expression of *myh6* in (A) control MO-injected and (B) *unc45b* MO (1)-injected embryos. (C, D) Expression of *vmhc* in (C) control MO-injected and (D) *unc45b* MO (1)-injected embryos. (E, F) Expression of *myl7* in (E) control MO-injected and (F) *unc45b* MO (1)-injected embryos.

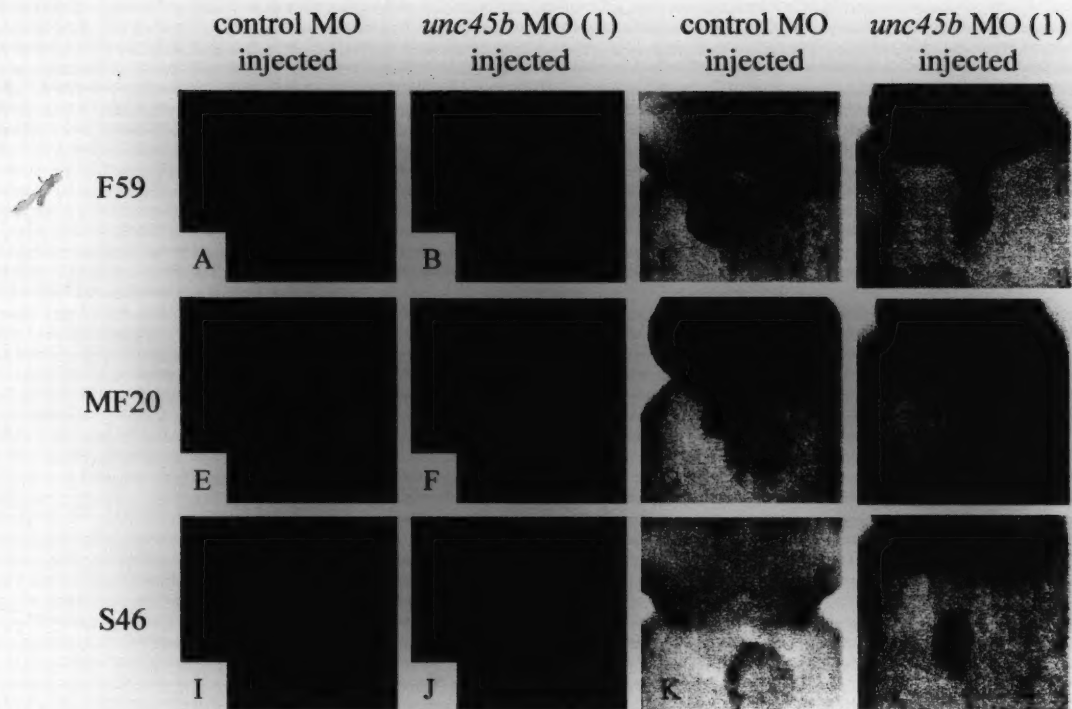


Figure 3.24 Protein localization of cardiac markers

Ventral views of 48 hpf embryos raised in 0.03% PTU with anterior to the top. (A-D) F59 immunostaining in the atrium and ventricle of (A,C) control MO-injected and (B,D) *unc45b* MO (1)-injected embryos. (E-H) MF20 immunostaining in the atrium and ventricle of (E,G) control MO-injected and (F,H) *unc45b* MO (1)-injected embryos. (I-L) S46 immunostaining in the atrium (I,K) control MO-injected and (J,L) *unc45b* MO (1)-injected embryos. (A,B,E,F,I,J) Immunostaining using fluorescent secondary antibodies and confocal microscopy at 20 x magnification. (C,D,G,H,K,L) Immunostaining using DAB detection system at approximately 20 x magnification.



Figure 3.25 Cardiac abnormalities in *unc45b* morphants

Lateral views with anterior towards the left of live embryos. (A-D) DIC photographs of a control MO-injected embryo with (A) atrium in plane of focus or (B) ventricle in plane of focus, (C) a *unc45b* MO (1)-injected embryo at 52 hpf and (D) a *unc45b* MO (1)-injected embryo at 3 dpf. (E-H) Brightfield image of a (E) control MO-injected embryo and a (F) *unc45b* MO (1)-injected embryo displaying cardiac edema at 3 dpf. (a; atrium, v; ventricle).

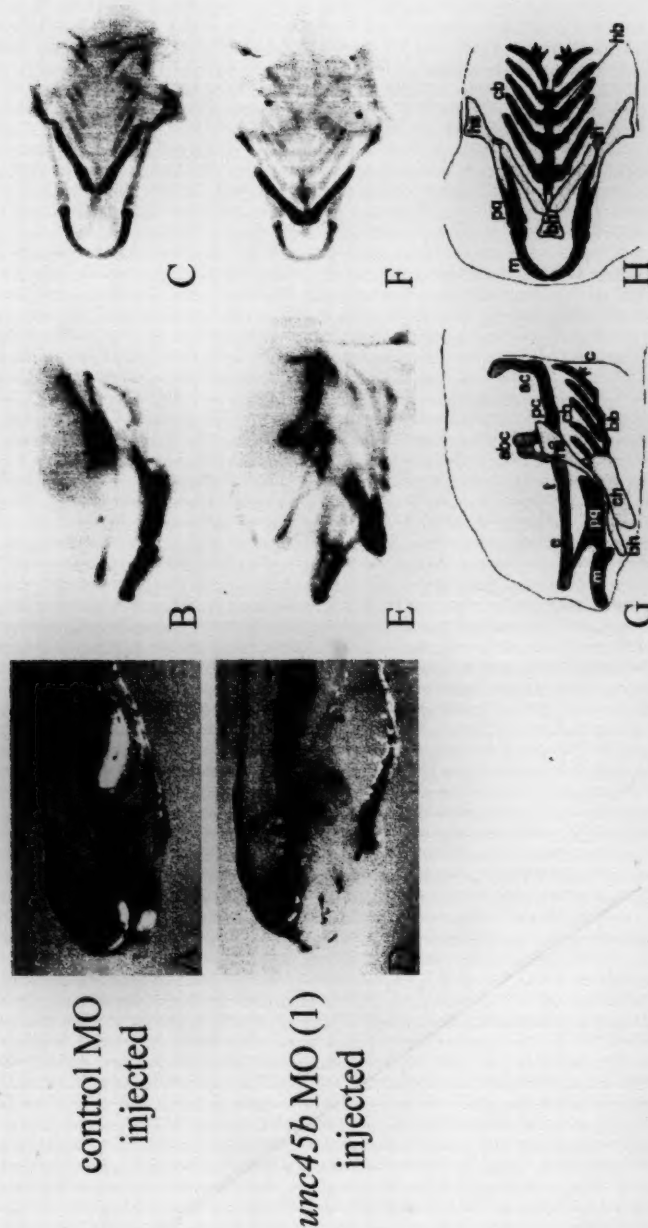


Figure 3.26 *unc45b* morphants display dysmorphic jaw structure at 5 dpf

(A, D) Lateral view with anterior to the left of a living larva injected with (A) control MO or (D) *unc45b* MO (1). (B-F) Alcian blue staining. (B, E) Lateral view with anterior to the left of (B) control MO-injected or (E) *unc45b* MO (1)-injected animals. (C, F) Ventral view with anterior to the left of (C) control MO-injected or (F) *unc45b* MO (1)-injected animals. (G, H) Diagrams of the patterns of larval jaw cartilages. (H) Ventral diagram. Cartilages of the same segment share the same color: P1 (mandibular, blue), P2 (hyoid, yellow), P3 (first branchial, pink), P4 (orange), P5 (green), P6 (purple), and P7 (black). The neurocranium is shaded uniformly grey. abc, anterior basicranial commissure; ac, auditory capsule; bb, basibranchial; bh, basihyal; c, cleithrum; cb, ceratobranchial; ch, ceratohyal; e, ethmoid plate; hb, hypobranchial; hs, hyosymplectic; ih, interhyal; m, Meckel's cartilage; n, notochord; ot, otic capsule; pc, parachordal; pq, palatoquadrate; t, trabeculae crani. (modified from Schilling *et al.*, 1996).

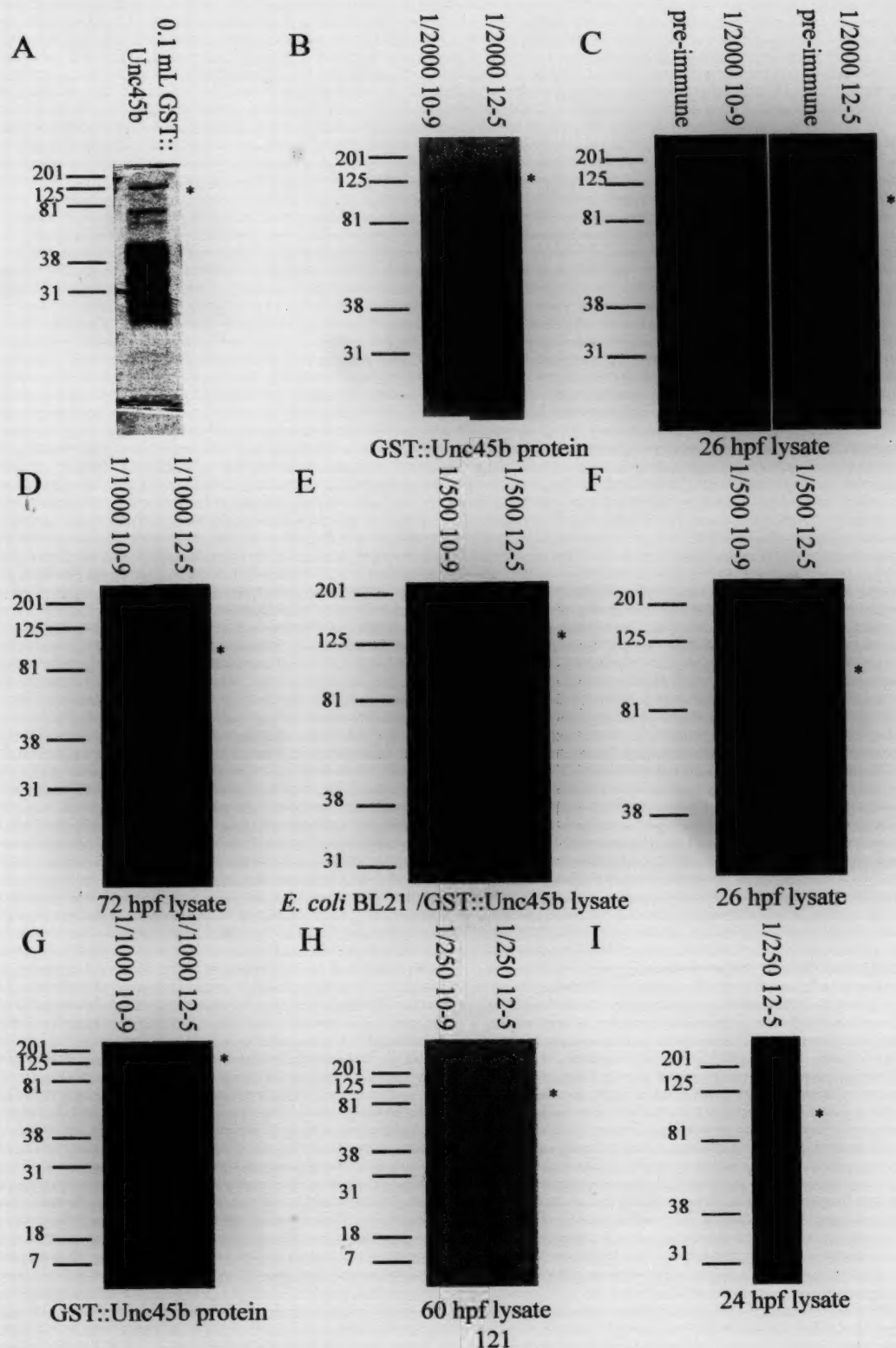


Figure 3.27 Expression of GST::Unc45b protein and antibody production (A) Coomassie stained SDS-PAGE gel showing presence of purified GST::Unc45b protein. Full length protein runs at expected size (131 kDa) but majority is degraded into smaller fragments. (B-I) Western analysis using (B) a 1/2000 dilution of test bleed 1 from rabbits 10-9 and 12-5 against purified GST::Unc45b protein demonstrating reactivity against the recombinant protein, (C) a 1/500 dilution of the pre-immune bleed and a 1/2000 dilution of the final bleed antisera from rabbits 10-9 and 12-5 against 26 hpf lysate, (D) a 1/1000 dilution of test bleed 3 from rabbits 10-9 and 12-5 following clearing by incubation with *E. coli* BL21(*DE3 pLysS*) acetone powder against 72 hpf zebrafish lysate, (E-F) a 1/500 dilution of antisera from rabbits 10-9 and 12-5 following purification by incubating with nitrocellulose bound with GST::Unc45b against (E) lysate of *E. coli* BL21(*DE3 pLysS*) cells expressing GST::Unc45b or (F) 26 hpf zebrafish lysate, (G) a 1/1000 dilution of antisera from rabbits 10-9 and 12-5 following purification by incubating with CNBr-activated Sepharose 4 Fast Flow (Amersham) coupled with GST::Unc45b against GST::Unc45b protein. This protein appears to be badly degraded and is therefore running at approximately 32 kDa, (H) a 1/250 dilution of antisera from rabbits 10-9 and 12-5 following purification by incubating with CNBr-activated Sepharose 4 Fast Flow (Amersham) coupled with GST::Unc45b against 60 hpf zebrafish lysate and (I) a 1/250 dilution of antisera from rabbit 12-5 following purification by incubating with CNBr-activated Sepharose 4 Fast Flow (Amersham) coupled with GST::Unc45b (aa 2-404) against 24 hpf zebrafish lysate. Standard sizes in kilodaltons are indicated to the left of each gel. Asterisks indicate approximate expected size of protein.

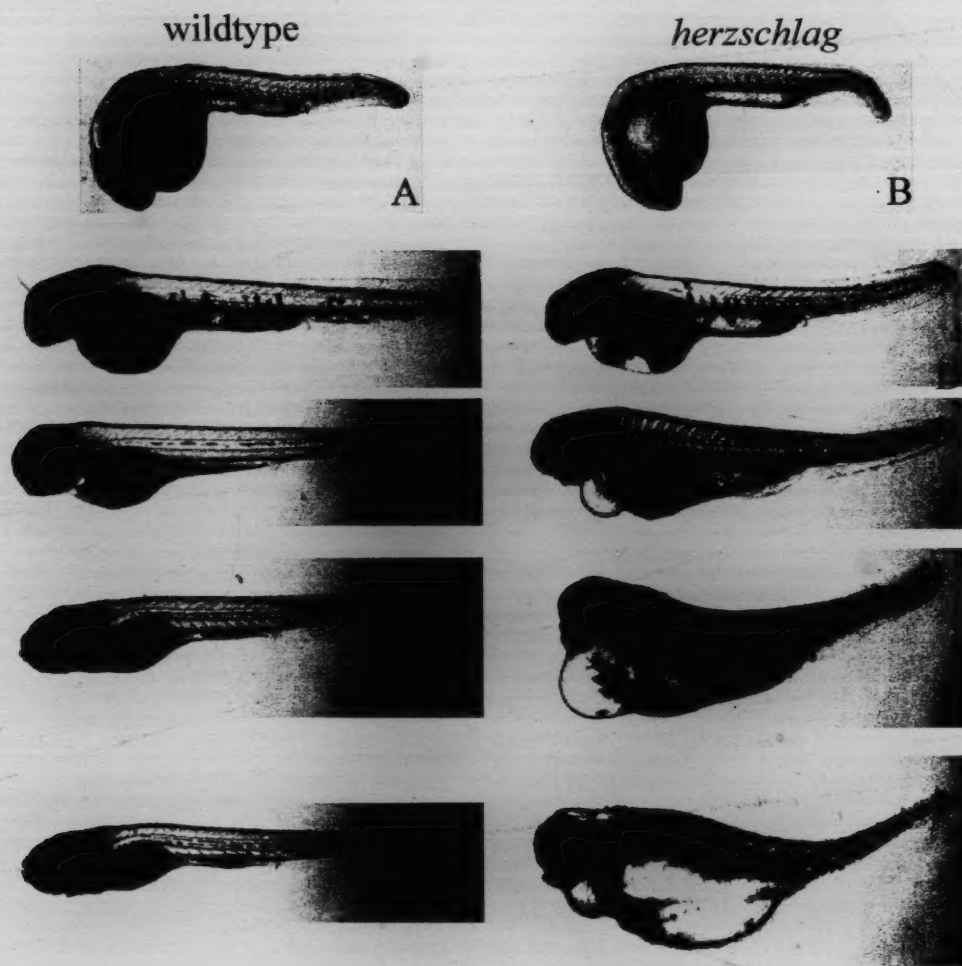


Figure 3.28 Comparison of wildtype and *herzschnag* animals
 Photographs of living (A,C,E,G,I) wildtype or (B,D,F,H,J) *herzschnag*
 larvae with anterior to the left at (A,B) 24 hpf, (C,D) 48 hpf, (E,F) 3 dpf,
 (G,H) 5 dpf and (I,J) 7 dpf. *herzschnag* larvae display severe cardiac and
 yolk sac edema.

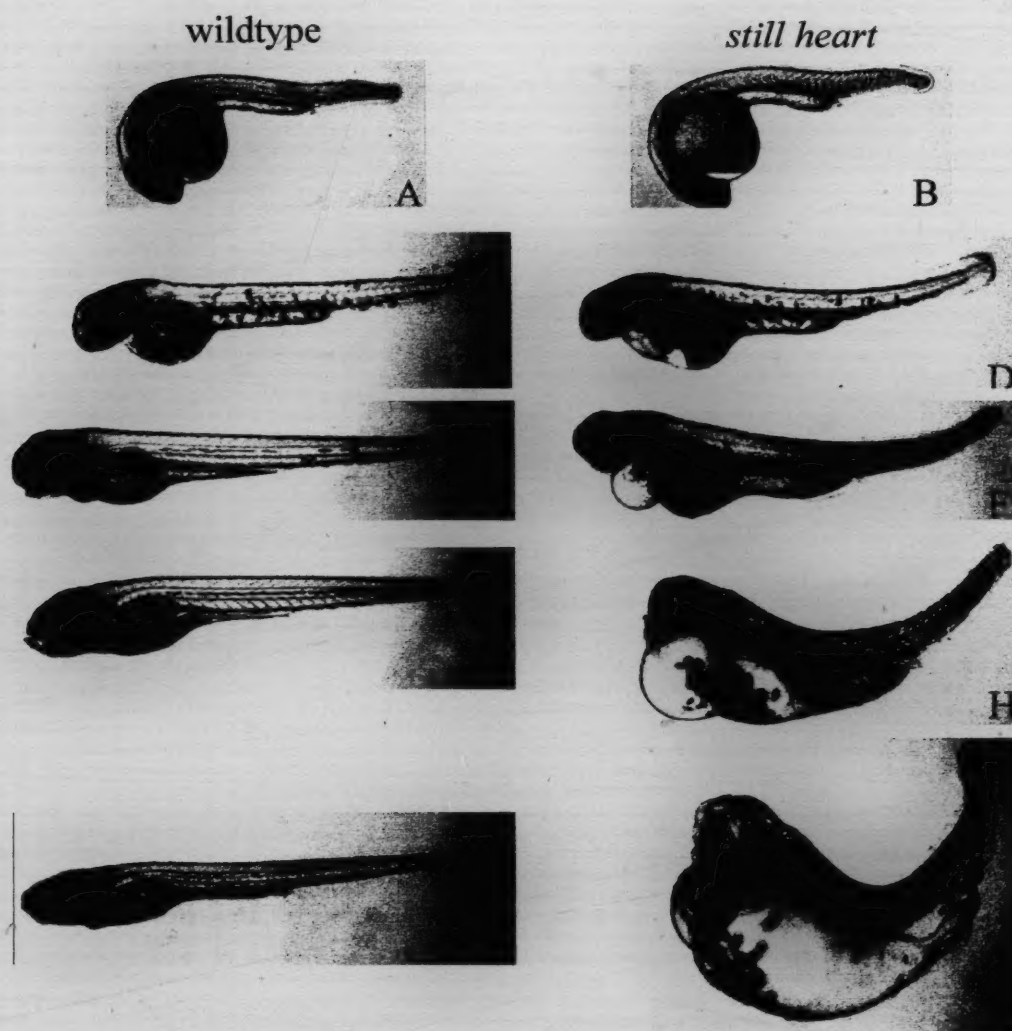


Figure 3.29 Comparison of wildtype and *still heart* animals
 Photographs of living (A,C,E,G,I) wildtype or (B,D,F,H,J) *still heart* larvae
 with anterior to the left at (A,B) 24 hpf, (C,D) 48 hpf, (E,F) 3 dpf, (G,H) 5 dpf
 and (I,J) 7dpf. *still heart* larvae display severe cardiac and yolk sac edema.

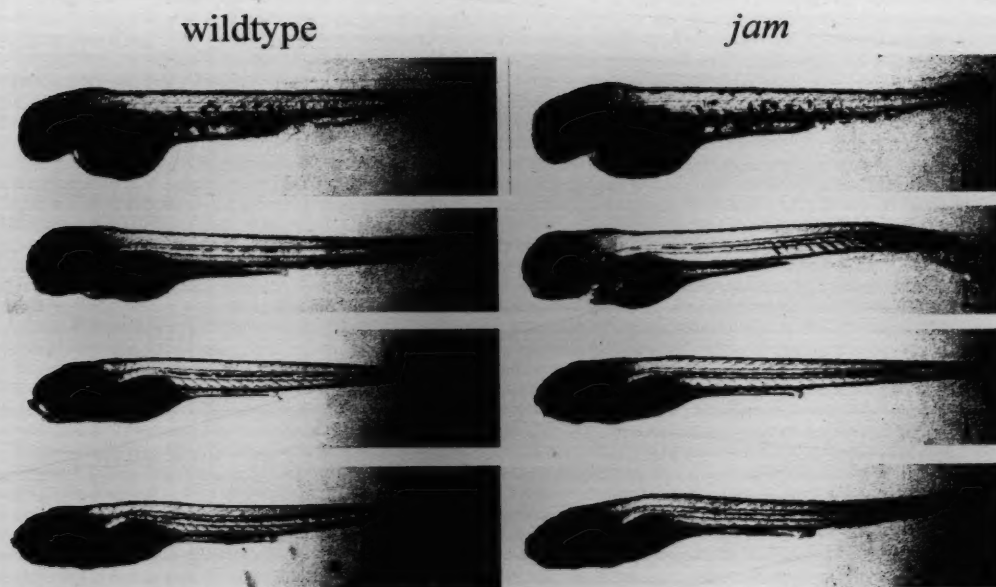


Figure 3.30 Comparison of wildtype and *jam* animals
 Photographs of living (A,C,E,G) wildtype or (B,D,F,H) *jam* larvae with anterior to the left at (A,B) 48 hpf, (C,D) 3 dpf, (E,F) 5 dpf, and (G,H) 7 dpf. On a gross level, *jam* animals are indistinguishable from wildtype.

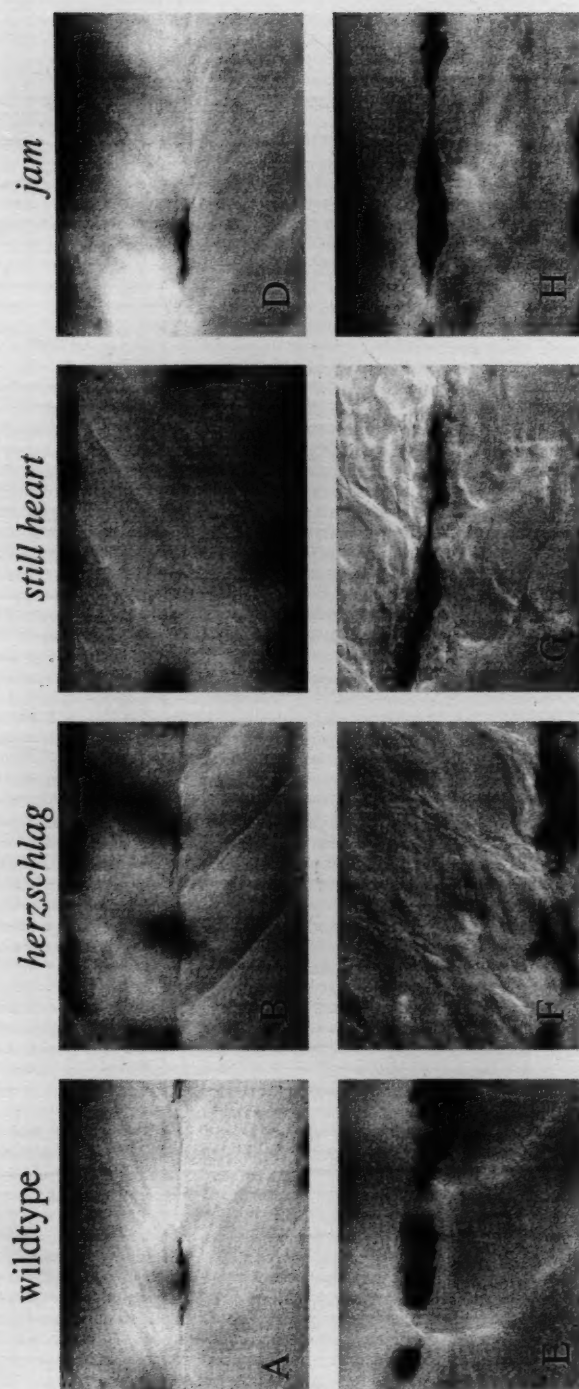


Figure 3.31 DIC photography of trunk muscle in mutant embryos

DIC photographs at approximately 63 x magnification of the trunk of living (A,E) wildtype (B,F) *herzschlag* (C,G) *still heart* and (D,H) *jam* embryos at (A-D) 48 hpf and (E-H) 5 dpf. Anterior is to the left. The muscle of *herzschlag* and *still heart* embryos lack visible striation at 48 hpf and develops vacuoles at 5 dpf. The muscle of *jam* embryos appears normal at 48 hpf but striation is not clear at 5 dpf.

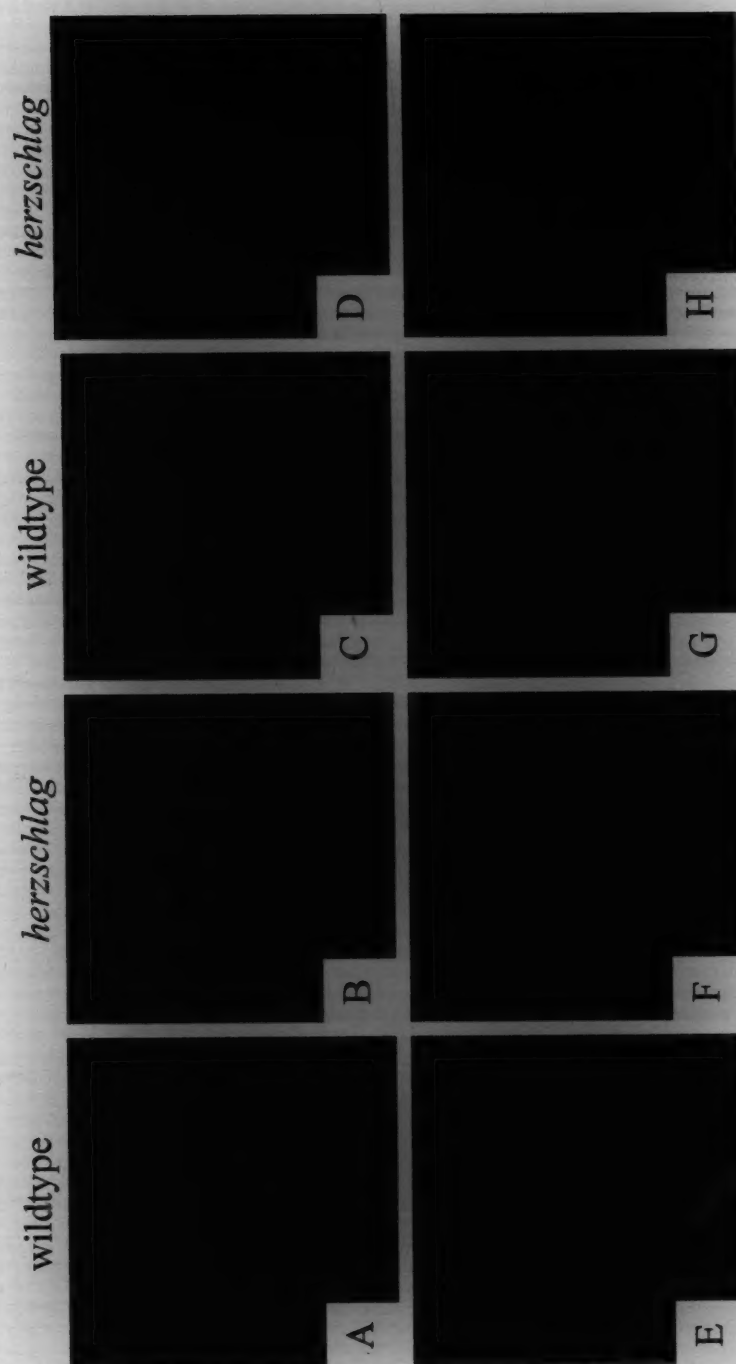


Figure 3.32 Immunostaining of *herzschnag* embryos with anti-myosin antibodies (A-D) 24 hpf embryos and (E-H) 48 hpf embryos with anterior to the left. (A,B,E,F) F59 staining of the trunk muscle in (A,E) wildtype and (B,F) *herzschnag* embryos. (C,D,G,H) S58 staining of the trunk muscle in (C,G) wildtype and (D,H) *herzschnag* embryos. Photographs were taken at (A-D) 63 x magnification and (E-H) 63x magnification with 4 x optical zoom.

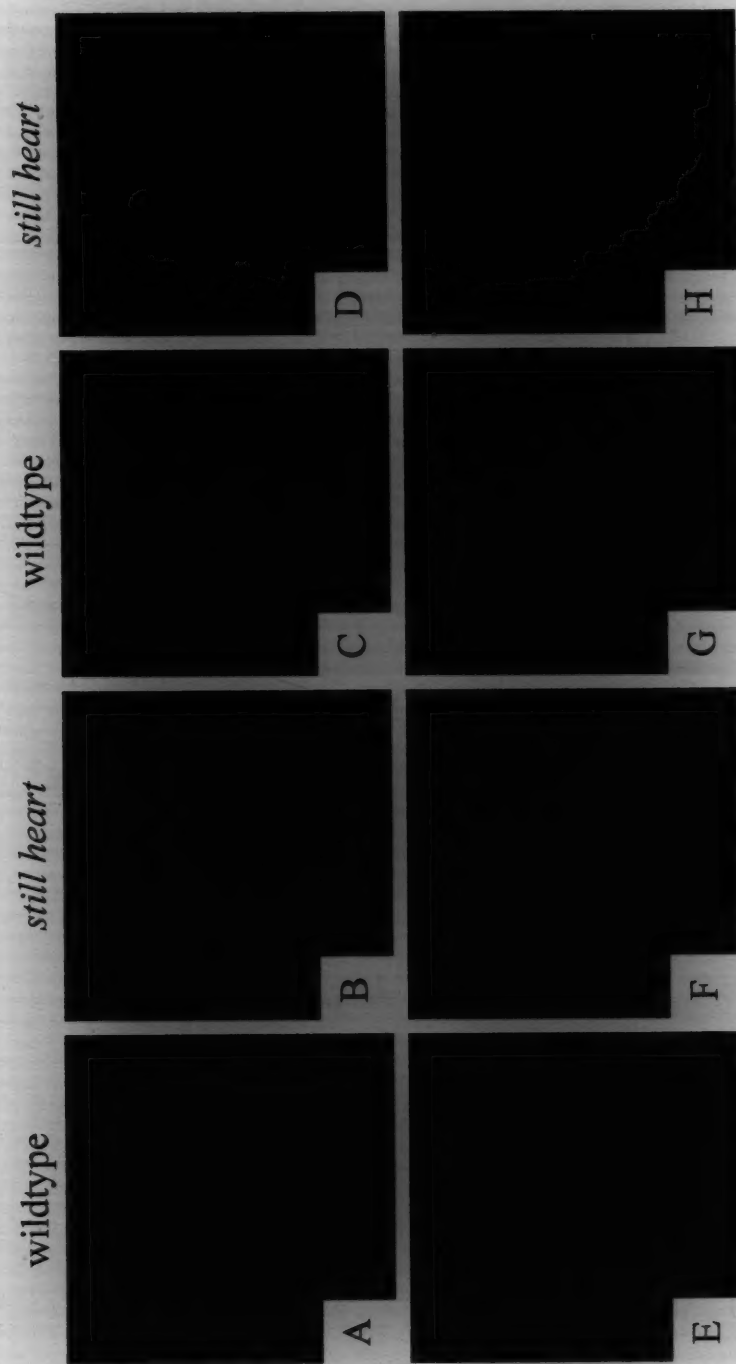


Figure 3.33 Immunostaining of *still heart* embryos with anti-myosin antibodies (A-D) 24 hpf embryos and (E-H) 48 hpf embryos with anterior to the left. (A,B,E,F) F59 staining of the trunk muscle in (A,E) wildtype and (B,F) *still heart* embryos. (C,D,G,H) S58 staining of the trunk muscle in (C,G) wildtype and (D,H) *still heart* embryos. Photographs were taken at (A-D) 63 x magnification and (E-H) 63x magnification with 4 x optical zoom.

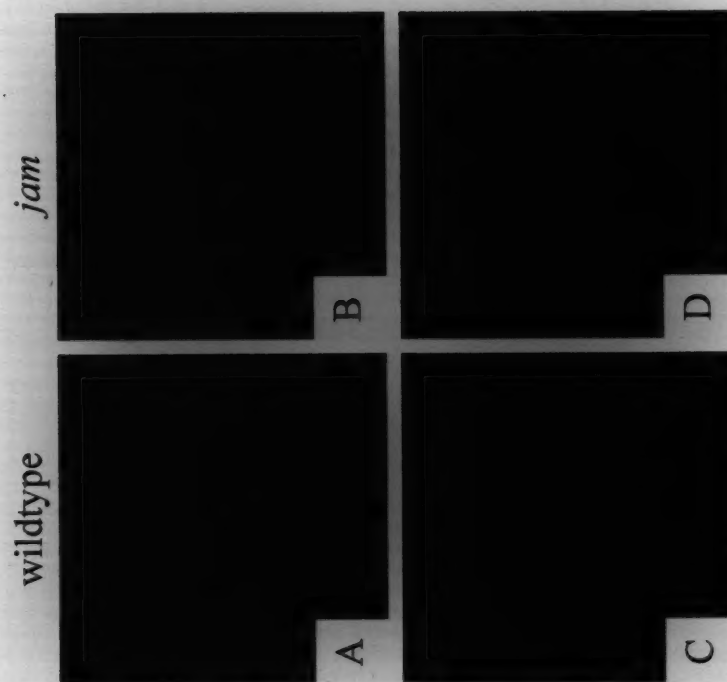


Figure 3.34 Immunostaining of *jam* embryos with anti-myosin antibodies 48 hpf embryos with anterior to the left. (A,B) F59 staining of the trunk muscle in (A) wildtype and (B) *jam* embryos. (C,D) S58 staining of the trunk muscle in (C) wildtype and (D) *jam* embryos. Photographs were taken at (A,B) 63 x magnification and (C,D) 63x magnification with 4 x optical zoom.

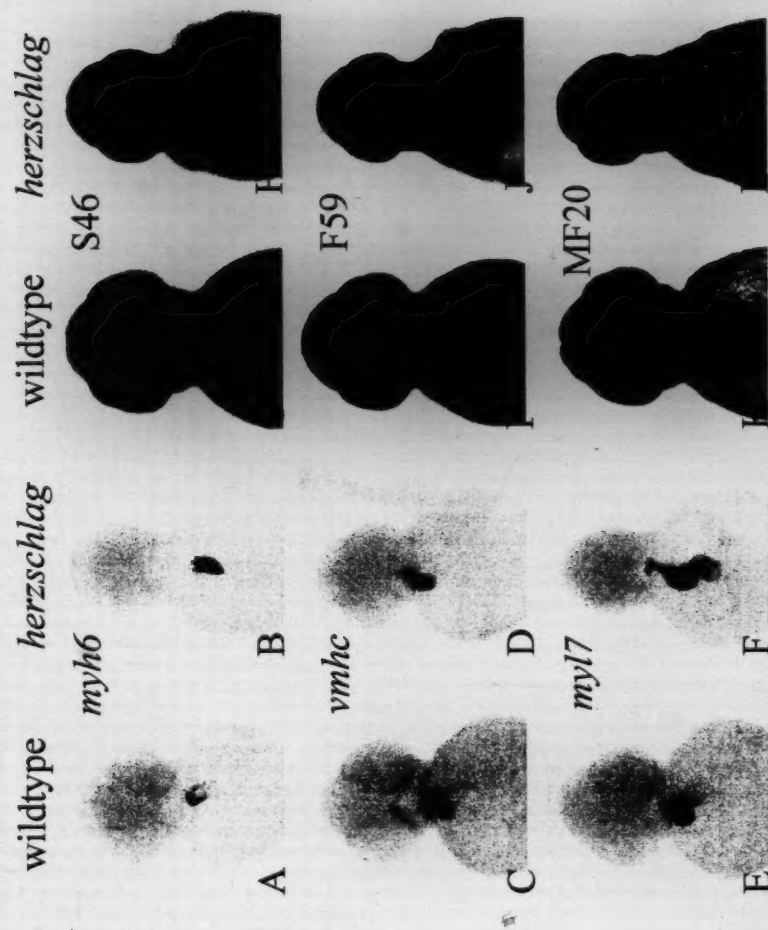


Figure 3.35 Analysis of cardiogenesis of *herzschnlag* embryos using cardiac *in situ* markers and antibodies. Ventral views of 48 hpf embryos raised in 0.003% PTU with anterior to the top. (A, B) Expression of *myh6* in (A) wildtype and (B) *herzschnlag* embryos. (C, D) Expression of *vmhc* in (C) wildtype and (D) *herzschnlag* embryos. (E, F) Expression of *myl7* in (E) wildtype and (F) *herzschnlag* embryos. (G, H) S46 antibody staining of the atrium in (G) wildtype and (H) *herzschnlag* embryos. (I, J) F59 antibody staining of the atrium and ventricle in (I) wildtype and (J) *herzschnlag* embryos. (K, L) MF20 antibody staining of the atrium and ventricle in (K) wildtype and (L) *herzschnlag* embryos.

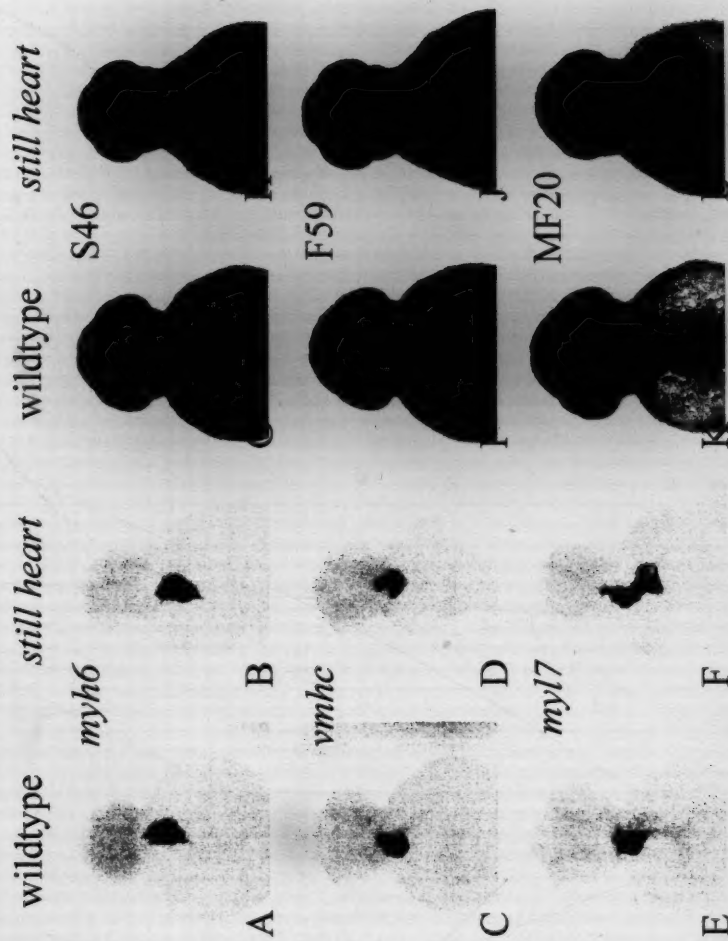


Figure 3.36 Analysis of cardiogenesis of *still heart* embryos using cardiac *in situ* markers and antibodies. Ventral views of 48 hpf embryos raised in 0.003% PTU with anterior to the top. (A, B) Expression of *myh6* in (A) wildtype and (B) *still heart* embryos. (C, D) Expression of *vmhc* in (C) wildtype and (D) *still heart* embryos. (E, F) Expression of *myl7* in (E) wildtype and (F) *still heart* embryos. (G, H) S46 antibody staining of the atrium in (G) wildtype and (H) *still heart* embryos. (I, J) F59 antibody staining of the atrium and ventricle in (I) wildtype and (J) *still heart* embryos. (K, L) MF20 antibody staining of the atrium and ventricle in (K) wildtype and (L) *still heart* embryos.

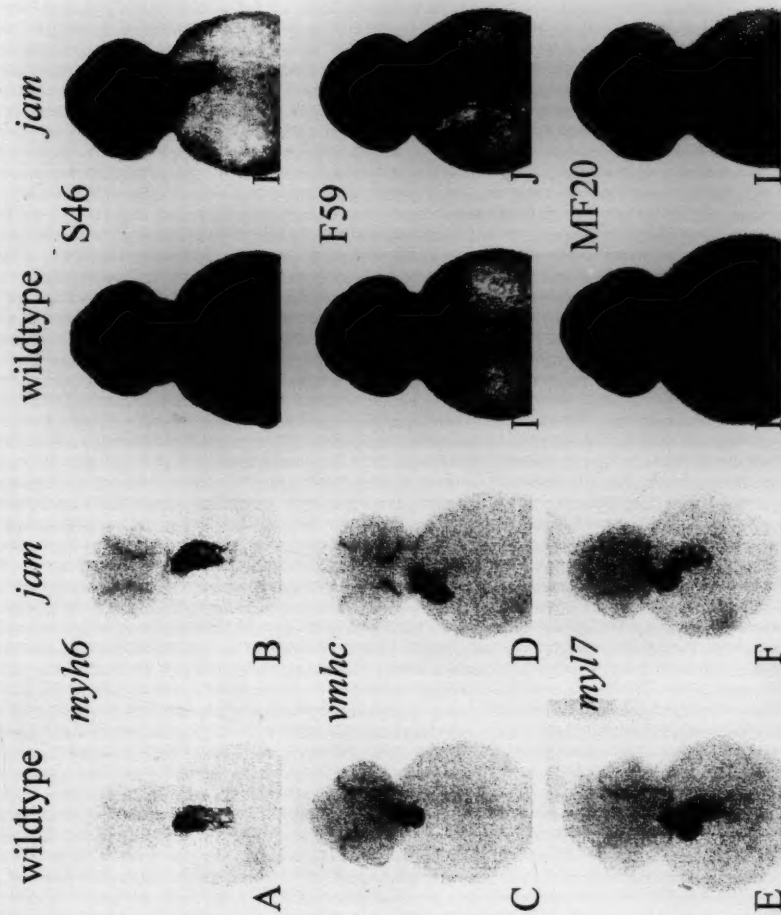


Figure 3.37 Analysis of cardiogenesis of *jam* embryos using cardiac *in situ* markers and antibodies. Ventral views of 48 hpf embryos raised in 0.003% PTU with anterior to the top. (A, B) Expression of *myh6* in (A) wildtype and (B) *jam* embryos. (C, D) Expression of *vmhc* in (C) wildtype and (D) *jam* embryos. (E, F) Expression of *myl7* in (E) wildtype and (F) *jam* embryos. (G, H) S46 antibody staining of the atrium in (G) wildtype and (H) *jam* embryos. (I, J) F59 antibody staining of the atrium and ventricle in (I) wildtype and (J) *jam* embryos. (K, L) MF20 antibody staining of the atrium and ventricle in (K) wildtype and (L) *jam* embryos.

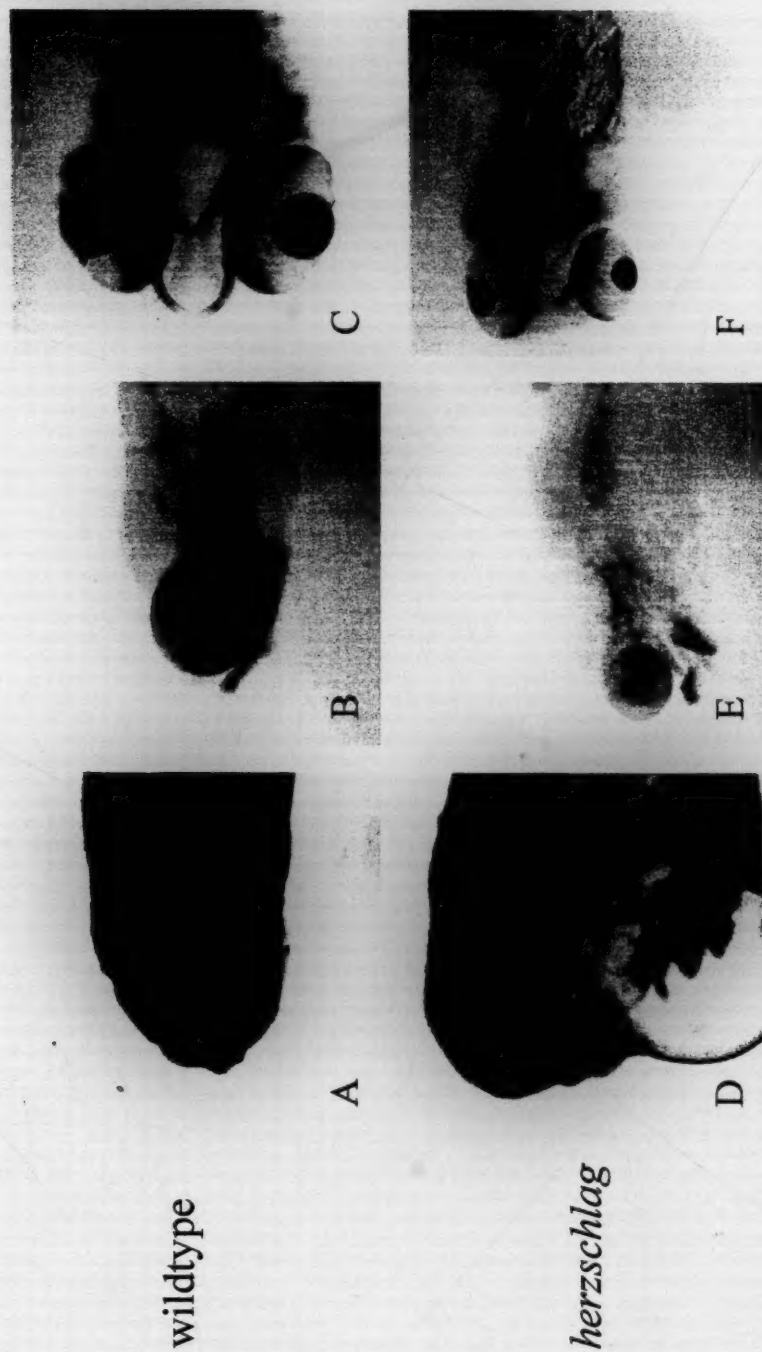


Figure 3.38 *herzschnag* mutant larvae display dysmorphic jaw structure at 5 dpf 5 dpf. (A, D) Lateral view with anterior to the left of a living (A) wildtype or (D) *herzschnag* larva. (B-F) Alcian blue staining. (B,E) Lateral view with anterior to the left of (B) wildtype or (E) *herzschnag* animals. (C,F) Ventral view with anterior to the left of (C) wildtype or (F) *herzschnag* animals.

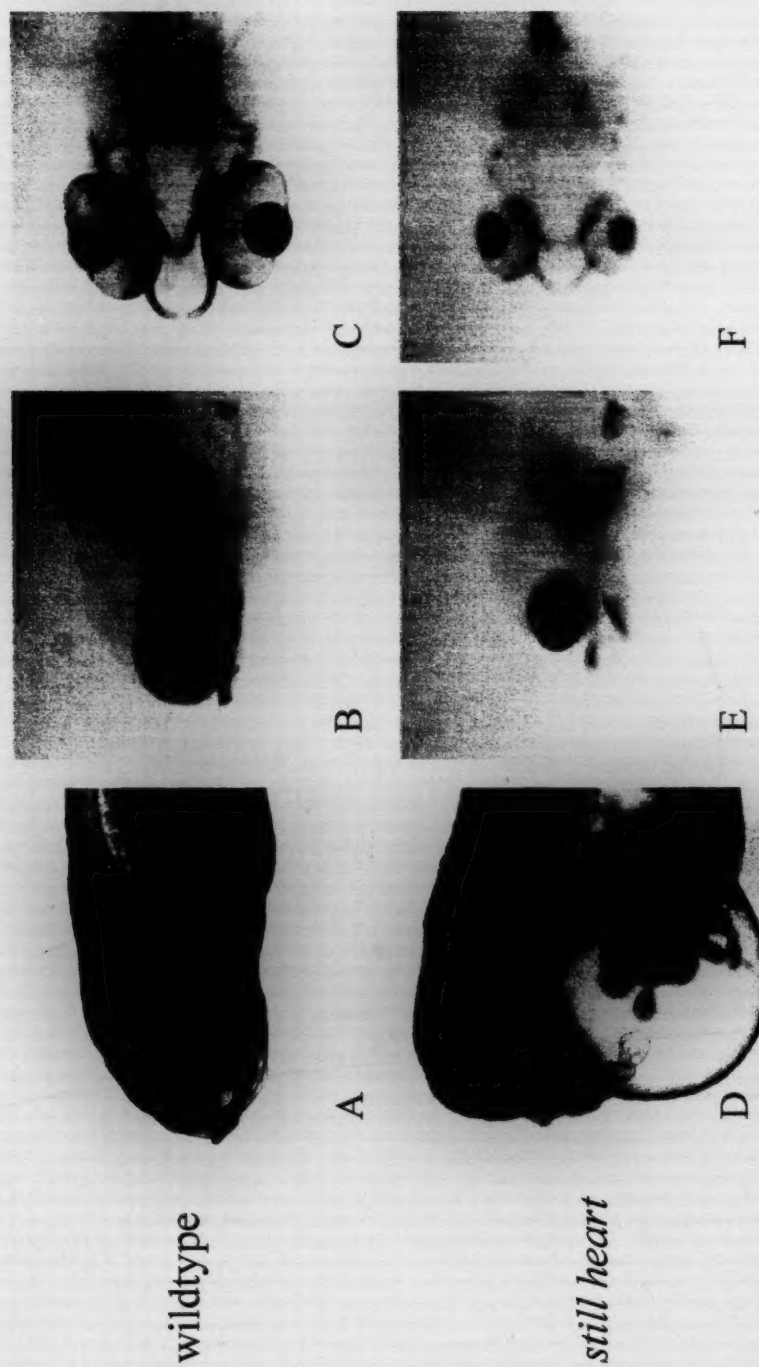


Figure 3.39 *still heart* mutant larvae display dysmorphic jaw structure at 5 dpf. (A, D) Lateral view with anterior to the left of a living (A) wildtype or (D) *still heart* larva. (B-F) Alcian blue staining. (B,E) Lateral view with anterior to the left of (B) wildtype or (E) *still heart* animals. (C,F) Ventral view with anterior to the left of (C) wildtype or (F) *still heart* animals.

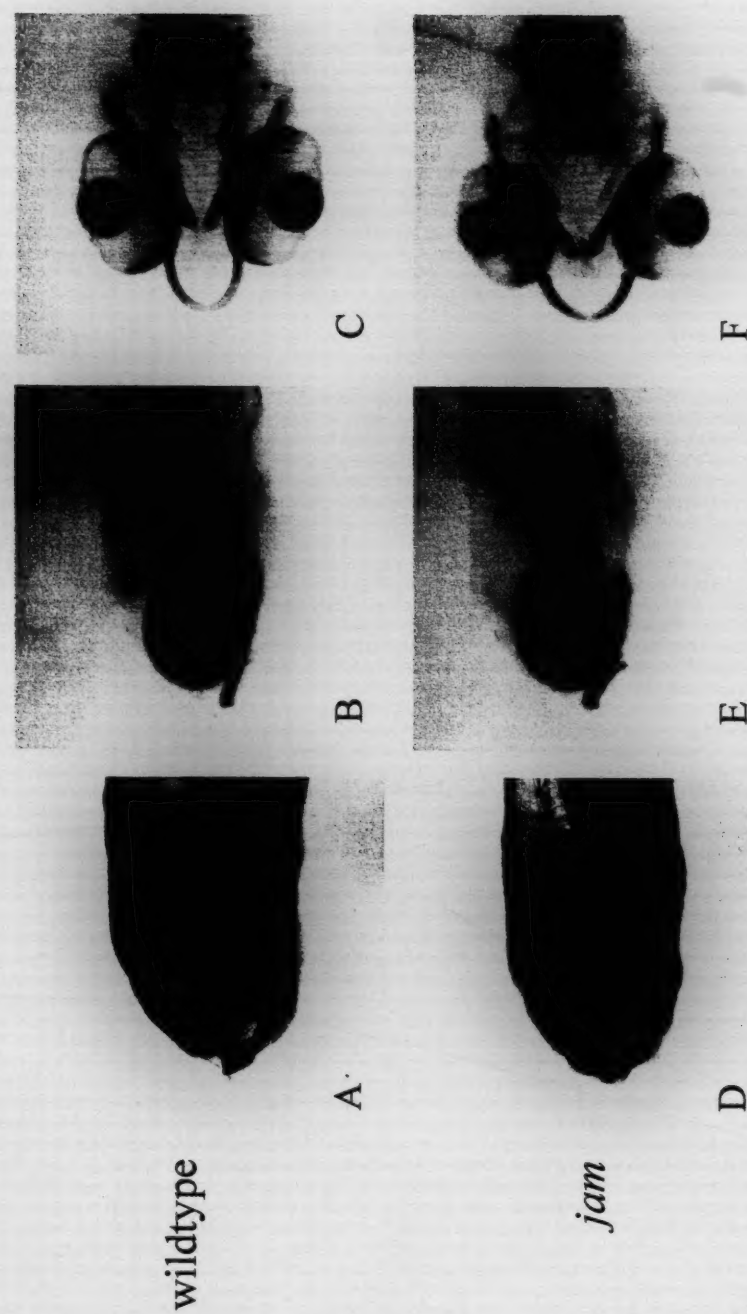


Figure 3.40 *jam* mutant larvae do not display jaw structure abnormalities at 5 dpf. (A, D) Lateral view with anterior to the left of a living (A) wildtype or (D) *jam* larva. (B-F) Alcian blue staining. (B,E) Lateral view with anterior to the left of (B) wildtype or (E) *jam* animals. (C,F) Ventral view with anterior to the left of (C) wildtype or (F) *jam* animals.

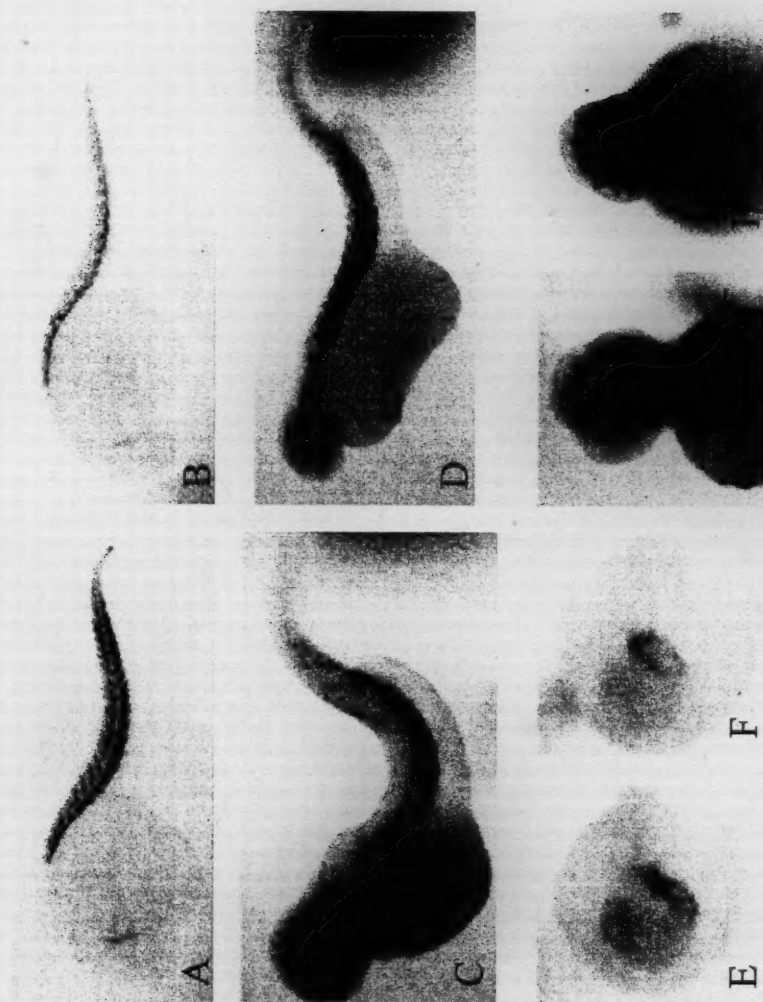


Figure 3.41 Expression of *unc45b* in *herzschnlag* embryos
 Expression of *unc45b* in (A,C,E,G) wildtype and (B,D,F,H) *herzschnlag* embryos at (A,B,E,F) 24 hpf and (C,D,G,H) 48 hpf. (A-D) Lateral view with anterior to left. (E,F) Dorsal view with anterior to left. (G-H) Ventral view with anterior to top.



Figure 3.42 Expression of *unc-45b* in still heart embryos
 Expression of *unc-45b* in (A,C,E,G) wildtype and (B,D,F,H) still heart embryos at (A,B,E,F) 24 hpf and (C,D, G,H,) 48 hpf. (A-D) Lateral view with anterior to left. (E,F) Dorsal view with anterior to left. (G-H) Ventral view with anterior to top.

4. Discussion

4.1 Why study Unc45 in zebrafish?

At the onset of my project, *unc-45* had only been studied in the nematode, *C. elegans*. Though *C. elegans* is a strong model organism its development does differ drastically from that of higher organisms. To determine if the knowledge gained about UNC-45 in *C. elegans* is relevant to mammalian development and could have medical implications, it had to be examined in vertebrate systems. For this reason, I decided to continue the study of *unc-45* in mammals and the zebrafish, *Danio rerio*. In order to study the effects of UNC-45 reduction in mammals, cell culture studies or knockout mice would have been needed. These techniques are costly and require expertise that was not available to me during my graduate studies. Additionally one could predict that this type of genetic manipulation would result in embryonic lethality, limiting its usefulness. However, by taking advantage of the external fertilization of zebrafish and morpholino oligonucleotides, I was able to phenocopy the effects of an *unc45b* mutation in zebrafish. This allowed observation of the defects that occur as a result of reducing Unc45b levels in live embryos. Additionally the availability of mutants acquired in a comprehensive screen for animals affected in their motility and cardiovascular system has proven advantageous in the study of striated muscle development in vertebrates as zebrafish muscle structure and development mirrors that of mammals (Taylor et al., 2003). For these reasons the majority of my research was conducted using zebrafish as a model organism.

4.2 *unc-45* is duplicated in vertebrate genomes

Vertebrates possess two homologues of *C. elegans unc-45*, termed *unc45a* and *unc45b*. Both of these maintain the same protein domain organization as *C. elegans* UNC-45 with each containing a TPR domain, a central domain and a UCS domain. These two genes are the only regions of the zebrafish genome with similarity to any of the *C. elegans* UNC-45 domains. The absence of additional genes or pseudogenes with similarity to *unc-45* suggests that they were not further duplicated as many other genes were following the divergence of teleosts from tetrapods or that, if they were duplicated, that the regions containing the duplicates have been since lost (Amores et al., 1998; Taylor et al., 2003).

unc45a and *unc45b* appear to have diverged significantly in respect to their expression and function. Though *unc-45* expression is restricted to muscular and germline tissues in *C. elegans*, one of the vertebrate homologues, *unc45a* shows a much broader expression pattern in mammals and zebrafish. Northern analysis demonstrates that in humans *UNC45A* is expressed in both the fetus and the adult in every tissue type examined, including those that do not have a muscular component. My data is consistent with the findings of Price *et al.* (2002) who show that this is also true in murine tissues. Examination of the expression pattern of *Unc45a* by *in situ* hybridization corroborates the northern analysis by also demonstrating that *Unc45a* is widely expressed but its expression appears to be enhanced in developing tissues such as the branchial arches, limb buds, brain and tail.

Consistent with the *in situ* pattern observed in mouse embryos, zebrafish *unc45a* appears to be expressed ubiquitously with enrichment in the vascular system. A

homozygous lethal null mutation has been identified in *unc45a* in *kurzschluss* mutants (B.L. Roman, personal communication). These mutants display a very specific phenotype associated with their vasculature as aortic arches 5 and 6 are perturbed in their connections with the ventral aorta that, in turn, results in circulatory problems. The role of zebrafish *unc45a* is unexpected with respect to published results regarding *UNC45A* in humans and *unc-45* in *C. elegans* embryos. In humans *UNC45A* appears to have a role in chaperoning the progesterone receptor complex (Chadli et al., 2006). This suggests that in non-muscle tissues *UNC45A* aids in folding proteins other than those from the myosin family leaving open the possibility of numerous as yet unidentified client proteins for *UNC45A*.

In *C. elegans* *UNC-45* is required to undergo cytokinesis due to the interaction between *UNC-45* and the non-muscle myosin, *NMY-2* (Kachur et al., 2004). Surprisingly this role of *Unc45* appears to be missing in zebrafish despite the fact that *NMY-2*'s role in cell division appears to be conserved between *C. elegans* and zebrafish (Urven et al., 2006). *Unc45a* or *Unc45b* may, in fact, be chaperoning *NMY-2* but any defects that would arise due to a reduction in their activity, either through mutation or through morpholino-targeted knockdown of expression, might be masked due to the contribution of maternal *unc45a* or *unc45b* RNA or protein to the early zebrafish embryo as will be discussed further below.

4.3 Comparison of *unc-45* phenotypes in *C. elegans* and zebrafish skeletal muscle.

4.3.1 *unc45b* expression in the zebrafish embryo

In zebrafish, *unc45b* appears to share its expression pattern with that of *C. elegans* *unc-45*, as it is expressed exclusively in tissues that will differentiate into striated muscle. These tissues include the presomitic mesoderm, adaxial cells, somites and the pectoral fin and cranial muscle. Using RT-PCR, I was also able to look at the *unc45b* expression at the transcriptional level at developmental time points where *in situ* hybridization results were inconclusive. This shows that *unc45b* RNA is maternally provided and, although this procedure does not give quantitative results, it appears that the level of *unc45b* decreases at approximately 6 hpf. It is possible that at this time the maternal transcript is depleted but zygotic expression of this gene has not yet been initiated. This shows conclusively that *unc45b* RNA is present in the zebrafish oocyte but leaves open the possibility that Unc45b protein is also maternally provided. In the absence of an antibody against Unc45b, however, this cannot be presently ascertained.

Because we do not have an antibody that reacted specifically against Unc45b, I attempted to mimic Unc45b localization by expressing a Myc::Unc45b fusion in zebrafish embryos. Although expression was achieved in skeletal muscle cells, the fusion protein was not localized to any specific structure. These results were inconclusive as the cytoplasmic localization observed may be due improper folding of the Unc45b protein in the presence of the Myc epitope. It is also possible that the entire fusion protein is not translated *in vivo*, although this possibility is unlikely as there were no truncation or frameshift mutations present in the *unc45b* coding region. Alternatively Unc45b may not

actually be localized to the sarcomere in zebrafish as it is in *C. elegans* (Ao and Pilgrim, 2000). It could be that the localization of UNC-45 to myosin-containing structures is unique to *C. elegans* as, with the exception of Rng3p in *S. pombe*, it has not been shown that any other UCS proteins localize to myosin-containing structures. Additionally Horlick (2005) has shown that the localization of UNC-45 to the thick filaments is not required for motility. Although in animals expressing chimeric myosin molecules UNC-45 did not localize to the sarcomere, they were still motile. This implies that UNC-45 may be present in the thick filaments only to monitor the folding state of the myosin molecules but that this localization is extraneous and that UNC-45 undertakes its function as a myosin chaperone in the cytoplasm of the muscle cells.

4.3.2 Phenotype of *unc45b* morphants

Using gene knockdown, I have demonstrated that Unc45b in zebrafish, as in *C. elegans*, is required for embryonic motility but not for muscle specification or differentiation. To determine if reducing Unc45a and Unc45b levels concurrently might recapitulate the cytokinetic defects observed in *C. elegans* RNAi embryos, morpholinos targeted to *unc45a* and *unc45b* were combined. However this does not worsen the phenotype. This is likely because morpholino oligonucleotides, acting post-transcriptionally, do not have the ability to reduce the maternal contribution of proteins.

The skeletal muscle of these paralyzed zebrafish embryos with decreased levels of Unc45b lack any obvious striation due to a reduction in myosin-containing filaments. This was observed by TEM and is consistent with UNC-45 possessing myosin-chaperone activity. While this paralysis is most likely due to a disorganization of the skeletal

muscle myosin it could also be attributed to a loss of actin organization. This is surprising because, to date, UNC-45 has only been shown to interact with myosin filaments. Likely the actin-containing thin filaments are still present but, in the absence of thick filaments, myofibrils do not properly assemble. Hence, it appears that there is also an absence of actin organization. As will be discussed below, I conclude that Unc45b is required *in vivo* for the proper assembly of myosin-containing structures in skeletal muscle.

In 24 hpf morphants, neither the F59 nor the S58 epitope, both of which represent myosin heavy chains (Crow and Stockdale, 1986; Devoto et al., 1996), appear to be properly organized as assessed by muscle striation. At 48 hpf, this remains true for the S58 epitope but the F59 epitope appears to have recovered some organization although these embryos were still immotile. This demonstrates that partial organization of the slow muscle is not sufficient for motility. The recovery of F59 organization suggests the activity of a second myosin co-chaperone, expressed later, that is able help this epitope recover its organization. Another possibility is that the S58 and F59 myosin epitopes are organized similarly to the two major myosins in the body wall muscle of *C. elegans*. In *C. elegans* muscle, MHC A is localized only to the central region of the thick filament and MHC B is localized to the distal regions of the thick filament (Miller et al., 1983). It has been shown that UNC-45 only co-localizes with and is only required for the organization of the major isoform, MHC B (Ao and Pilgrim, 2000). In zebrafish slow muscle, S58 has a more restricted localization pattern and may be equivalent to MHC A whereas the F59 epitope seems to be a major protein component of the zebrafish thick filament and has a more similar localization pattern to MHC B. In zebrafish it appears

that myosin heavy chain recognized by S58 has a higher dependency on Unc45b for organization than the F59 epitope, contrasting what has been observed in *C. elegans*. This demonstrates that Unc45b is required for slow muscle myosin organization. It could be presumed that the same could be said for fast muscle but this cannot be said conclusively as fewer resources are yet available to study fast muscle myosins in zebrafish.

4.4 Unc45b in other striated muscle.

4.4.1 Unc45b has a role in zebrafish cardiac development.

In addition to the paralysis seen in *unc45b* morphants, I also show a previously unrecognized role for UNC-45 in cardiac development. Initially *unc45b* is expressed in both chambers of the developing heart but is eventually restricted to the ventricle. When Unc45b levels are reduced, the ventricle fails to contract while the atrium remains contractile. In zebrafish, the two chambers of the heart express distinct myosin heavy chains, an atrium specific myosin heavy chain (*myh6*) and a ventricle-specific myosin heavy chain (*vmhc*) (Berdougo et al., 2003; Yelon et al., 1999). There are at least three possible explanations for the differences seen between the atrium and the ventricle. First, it is possible that only the ventricular myosin heavy chain folding is dependent on the chaperone activity provided by UNC-45. Second, the threshold level of UNC-45 required in the ventricle may be higher than that of the atrium and there is still sufficient residual UNC-45 activity in the atrium of the morphant embryos to maintain function.

Finally, the atrium may be able to compensate for the lack of UNC-45 in the morphants by increasing expression of other cardiac molecular components. Supporting the latter possibility, the myosin heavy chain(s) recognized by F59 and MF20 are upregulated in the atrium of morphant embryos. We also see higher levels of *myl7*, a cardiac myosin light chain, at the transcriptional level. Berdougo *et al.* (2003) demonstrated a similar atrial enrichment of *myl7* in *weak atrium* mutants. Perhaps, under stress (related, in this case, to a reduction in *Unc45b* levels) increasing expression of these components in the atrium is sufficient to maintain function. Alternatively the ventricle may be under more stress because its contraction is responsible for circulating the blood throughout the embryo. It has also been shown that circulation is not impeded in *weak atrium* mutants despite the elimination of the atrial myosin heavy chain implying that ventricular function has a more important role in circulation (Berdougo *et al.*, 2003). Also ventricular formation and function appears to be particularly susceptible to mutations affecting the heart as it is more often affected than the atrium in genetic screens for cardiovascular mutants (Alexander *et al.*, 1998; Stainier *et al.*, 1996).

Zebrafish are an ideal model for studying early cardiac development because, as embryos and small larvae, they do not require a functioning circulatory system to supply oxygen to the developing tissues. However, this is not to say that zebrafish embryos do not require circulation. *unc45b* morphants display severe cardiac and yolk sac edema, common in embryos with cardiac defects (Garrity *et al.*, 2002; Szeto *et al.*, 2002; Walton *et al.*, 2006). This is likely secondary to circulatory problems. Kidney morphogenesis is dependent on blood flow through a mechanism involving the stretch-responsive *matrix metalloproteinase-2* (Serluca *et al.*, 2002) and because kidneys are responsible for

maintaining osmotic regularity, disruption of their development would result in the observed edema. *Unc45b* is also required for cardiac looping. This defect may also be secondary either to the lack of mechanical forces that circulation provides (Hove et al., 2003) or because morphants lack early myocardial function (Bartman et al., 2004). The roles of hydrodynamic and contractile forces in cardiac morphogenesis are still poorly understood, but the failure of cardiac looping in *unc45b* morphants is further evidence of its importance.

4.4.2 Requirement of *Unc45b* in craniofacial development.

Several craniofacial cartilages are displaced and/or malformed in *unc45b* morphants suggesting that striated muscle function is also necessary for establishing and/or maintaining proper jaw structure. This may be explained by the interdependent development of the cartilaginous structures and jaw musculature (Schilling and Kimmel, 1997). More likely, the maintenance or development of proper jaw structure is reliant on muscle function. Similar phenotypes have been observed previously the zebrafish strains, *grossmaul*^{m643} and *to273c* but the causal genetic loci have not yet been identified in these cases (Neuhauss et al., 1996; Schilling et al., 1996). It is likely that these also encode proteins required for muscle function. This aspect of the *unc45b* morphant phenotype is interesting because, in human myopathies, joints can fuse *in utero* due to lack of muscle contraction (Sung et al., 2003a; Sung et al., 2003b). Here it appears that structures can also separate because of problems with muscle contraction. In the absence of proper craniofacial muscle function, it appears that the cartilages are not able to adopt or maintain their proper position. This implies that jaw morphogenesis relies on the

physical contraction of this muscle to hold the cartilages in their compact arrangement and that in the absence of Unc45b they are displaced ventrally.

4.5 Is Unc45b a molecular myosin chaperone?

These results from zebrafish are consistent with the conservation of *unc-45* function as a myosin organizer or co-chaperone across metazoan species. Zebrafish *unc45b* shares a similar expression pattern with one of the *hsp90* homologues in zebrafish, *hsp90α*. *Hsp90α*, like *unc45b*, is expressed in the somites and pectoral fin muscle but lacks expression in the heart (Sass et al., 1999; Sass et al., 1996). Recently it was shown that zebrafish Unc45b can interact with Hsp90 and that knockdown of Hsp90α resembles the phenotype observed with loss of Unc45b activity (Etard et al., 2007). This adds credence to the *in vitro* studies implicating *unc-45* as a co-chaperone for myosin folding with Hsp90, at least in skeletal and fin muscle (Barral et al., 2002). Although Hsp70, Hsp90 and the chaperonin containing TCP-1 (CCT) have been shown to be involved in the folding of type II myosins, none are sufficient to attain the mature structure of the myosin motor domain (Srikakulam and Winkelmann, 1999; Srikakulam and Winkelmann, 2004). This research shows that the addition of muscle-specific factors is required to attain myosin's functional conformation. Unc45b is likely one of these required muscle-specific factors.

Without biochemical studies I have not shown directly that Unc45b is acting as a myosin chaperone or co-chaperone but the results I show support this conclusion. I speculate that the slow muscle myosin epitopes recognized by F59 and S58, as well as the

cardiac myosin heavy chains, particularly the ventricular myosin heavy chain are potential targets of the chaperone activity of *Unc45b* as each is affected by the reduction of *Unc45b* levels. This does not however mean that these are the only targets of *Unc45b* activity. Myosins expressed in the fast muscle, pectoral fin muscle and cranial muscle are also likely potential clients of *Unc45b*.

4.6 Potential causative mutations in *herzschlag*, *still heart*, and *jam*

Despite the obvious similarities in phenotype between *herzschlag* and *still heart* embryos and *unc45b* morphants, I have determined by sequencing and *in situ* hybridization that *unc45b* is not affected in these mutant strains of zebrafish. This leaves open the question then of what are the mutations that are causing the phenotypes of these fish, as well as the *jam* strain of fish? Each strain could be affected in any of the numerous components of the zebrafish sarcomere as they all show some degree of muscle disorganization, though *jam* embryos are affected to a much lesser degree than *herzschlag* or *still heart* embryos. It should be noted that I did not examine the *unc45b* sequence in *jam* embryos so it is still possible that *unc45b* mutations are present in this strain. However based on the differences in phenotype between *jam* homozygotes and *unc45b* morphants, I feel that this is unlikely.

Because *herzschlag* and *still heart* homozygotes are affected in both their skeletal muscle function and in their cardiac function, it is necessary that the gene affected in these strains must be expressed in both of these structures. Luckily the *still heart* mutation has been shown to be linked to chromosome 8 so I was able to search for

identified candidate genes on this chromosome that are expressed in both the somites and the heart (www.zfin.org). Of particular high interest were *smyd1b*, *obscurin* and *atp2a2a* as all are expressed in the skeletal muscle and cardiomyocytes.

Smyd1 is a histone methyltransferase known to be required for maturation of cardiac cells in mice and zebrafish while obscurin is a sarcomeric component required for M-line assembly (Gottlieb et al., 2002; Raeker et al., 2006; Tan et al., 2006; Waterston et al., 1980). Despite their differing molecular functions, morpholino-mediated knockdown of either of these proteins results in massive disorganization of the sarcomere. Reduction of Smyd1 results in paralysis and a lack of heartbeat; reduction of obscurin slows the heart rate (no motility phenotype was discussed). These results suggest that *smyd1b* is the strongest of the candidate genes possibly affected in *still heart* mutants. Another candidate, Atp2a2a, is a sarcoplasmic reticulum protein involved in the transport of calcium that regulated muscle contraction. In zebrafish reduction of Atp2a2a results in weak contraction of the heart and lethality at approximately 6 dpf (Ebert et al., 2005). A homologue of this gene exists, *atp2a1*, that is expressed exclusively in the skeletal muscle (Gleason et al., 2004). If *still heart* is affected at the *atp2a2a* locus, it is possible that *atp2a1* is upregulated in the skeletal muscle and compensates for loss of Atp2a2a function. This would account for the recovery of some motility in *still heart* embryos after 48 hpf. A final candidate gene situated on chromosome 8 is *ufd1-l* (ubiquitin fusion degradation 1-like). While the expression pattern of this gene is not known, the possibility exists that this gene could be involved in muscle development. *C. elegans* UNC-45 levels are regulated by the addition of ubiquitin moieties, a process involving UFD-2 (Hoppe et al., 2004). Improper regulation of these levels results in motility

problems due to muscle disorganization (Landsverk et al., 2007). Therefore it is possible that, in zebrafish, Unc45b levels are in part controlled by Ufd1 and a mutation in this gene, potentially the *still heart* mutation, could result in improper amounts of Unc45b present in the muscle cells thereby resulting in paralysis, cardiac dysfunction and dysmorphic craniofacial structures due to thick filament disorganization.

4.7 Future Directions

Identification of vertebrate Unc45b-interacting proteins

In order to fully understand why reducing Unc45b levels in zebrafish results in paralysis and cardiac dysfunction, the mechanism by which Unc45b acts must be further examined. One step in this process is to determine which proteins are physically interacting with Unc45b. To accomplish this biochemical studies must be pursued. Etard *et al.* (2007) recently demonstrated an interaction between Unc45b and Hsp90. Based on this and on previous studies in *C. elegans* and fungi I would predict that numerous myosin proteins are interacting with Unc45b but data from *C. elegans* suggest that UNC-45 is selective in its client proteins. In *C. elegans* muscle MHC A and MHC B are both present in the thick filament however UNC-45 only appears to be required for MHC B function (Ao and Pilgrim, 2000). This suggests that UNC-45 is not involved or is less involved in the folding of some myosins. Therefore it would be interesting to determine which zebrafish myosins are dependant on Unc45b to reach their mature conformation. One standard technique that screens for interacting proteins, the yeast 2-hybrid screen, would likely be unsuccessful at identifying Unc45b client proteins due to the limitations

of the system and the complexity of the myosin molecule. In order to properly fold, type II myosins found in muscle cells require muscle specific factors and, while Unc45b would potentially aid in the folding process, it likely does not act alone (Srikakulam and Winkelmann, 1999). Therefore these molecules would likely form insoluble aggregates in the yeast cells. Additionally myosins are cytoplasmic proteins and the yeast 2-hybrid system relies on the transport of the "bait" and the "prey" proteins into the cells nucleus. These aspects of the yeast 2-hybrid system likely contribute to the fact that, despite the strong evidence that *C. elegans* UNC-45 is interacting with MHC B, this interaction was not identified in an attempt to discover UNC-45-interacting proteins (Ao and Pilgrim, 2000).

In order to identify Unc45b client proteins, a new antibody specific to Unc45b should be developed. This would enable us to determine whether or not Unc45b localizes to the thick filaments in zebrafish and, via co-immunoprecipitation, allow us to identify proteins that interact with Unc45b *in vivo* in the zebrafish system.

Determine causative mutations in herzschnag, still heart and jam strains

Work is currently in progress in our lab to map the genes affected in the *herzschnag*, *still heart* and *jam* mutants. I have crossed these strains to the WIK strain, which is used for PCR-based mapping in zebrafish and have identified the resulting heterozygotes. To map these mutations polymorphisms in PCR fragment length can be used to determine the locations of the causative mutations (Bahary et al., 2004). Once candidate genes are identified corresponding BACs or cDNAs can be injected into embryos to attempt to rescue the mutant phenotype. To conclusively identify the

mutations causing the phenotypes in these strains, sequence of the cDNAs or the genomic regions containing these genes should be obtained. These results would potentially identify additional components of the zebrafish musculature and provide additional models of human skeletal myopathies and cardiomyopathies.

It should again be noted that the phenotype of the *jam* mutant observed was not as severe as predicted based of preliminary characterization (Chen et al., 1996). One explanation for this is that the acquired strain has accumulated background mutations that mask the original phenotype. Backcrosses to the Tu strain should be performed to eliminate these potential background mutations uncover the *jam* phenotype. If a more severe phenotype does appear following backcrossing, the resulting fish should be crossed to the WIK strain to more easily identify homozygous mutants which is necessary for PCR-based mapping strategies.

Use of steif mutant

Recently a strain of zebrafish fish, *steif*, was identified that was determined to be a null-allele of *unc45b*, resulting in the replacement of cysteine at position 788 with a stop codon (Etard et al., 2007). The identification of this mutant opens doors for areas of research that are not possible using morpholino-mediated knockdown techniques. Because this mutation creates a premature stop, it can be used to do domain analysis with regards to protein interactions. If Unc45b-interacting partners were identified, as discussed above, we could use the *steif* mutant to determine which, if any, additional proteins or which proteins no longer with the truncated protein. This would allow us to identify which specific proteins interact with this region of the UCS domain in zebrafish.

Genetically, *steif* could be crossed to other mutants known to have muscular defects (discussed further below) as a complementation screen to reveal additional *unc45b* mutants. If *steif* heterozygotes were crossed to other strains heterozygous for new *unc45b* mutations a quarter of the resulting embryos would present with the mutant phenotype. However if a non-allelic strain were used, all the progeny would be motile.

Steif heterozygotes could also be crossed to the *unc45a* mutant, *kurzschluss*, to create double mutants. This may eliminate some of the questions raised by potential redundancy between the zebrafish Unc45 proteins and expose a cytokinetic role in zebrafish similar to that seen in *C. elegans* (Kachur *et al.*, 2004). However, as discussed in section 4.2, maternal contribution of *unc45* RNA or protein may mask this role. To eliminate maternal Unc45 and bypass the lethality of *steif* and *kurzschluss* homozygosity, maternal-zygotic mutant fish could be produced. To accomplish this, primordial germ cell (PGC) development of wildtype animals is blocked via morpholino injection and germ cells derived from *steif*, *kurzschluss*, or a double mutant are used to replace the wildtype cells (Ciruna *et al.*, 2002). This would create embryos devoid of Unc45 activity and uncover any role *unc45* may play in early zebrafish development.

Screen for additional unc45b mutations in zebrafish

Although one strain of fish has been identified that carries a mutation in *unc45b*, there are still many strains of zebrafish that, based on initial phenotypic characterization, may also be affected in *unc45b*. These strains may contain null mutations in other Unc45 domains or may contain "leaky" mutations. These mutations would be helpful in studying the role of Unc45b in vertebrates. Currently there are at least 16 identified

mutants that show reduced motility and reduced muscle striation that have not yet been linked to any causative mutation and at least 21 mutants affected in their cardiac contractility similar to *unc45b* morphants (Chen et al., 1996; Granato et al., 1996; Stainier et al., 1996). *Heart attack*, *slinky*, *slop* and two unnamed mutants are said to be affected in both motility and cardiac function, similar to *herzschlag*, *still heart* and *jam*. These strains are also good candidates for *unc45b* mutations. However fish containing *unc45b* mutations need not be affected in both motility and cardiac function so one should not discard mutants affected in either one of the processes alone. Mutations affecting tissue specific expression or function of *unc45b* could result in immotility but not cardiac dysfunction or vice versa. This type of mutant would be useful in characterizing the tissue specific roles of Unc45b.

Recently TILLING (Targetting Induced Local Lesion IN Genomes) has become a popular method of identifying mutations in genes of interest in zebrafish (Amsterdam and Hopkins, 2006; Draper et al., 2004; Stemple, 2004). Here sperm are mutagenized and screened for mutations in the gene of interest through sequencing or misannealing of PCR products. This is a high throughput reverse genetic technique that may be useful in identifying additional mutations in the *unc45b* coding region.

Screen for UNC45B mutations in myopathy patients

To date, no mammalian model has been developed for studying the role of UNC-45 in development and no mutations in the human locus have yet been identified. We predict that null mutations of human *UNC45B* would be embryonic lethal due to defects in cardiac function but leaky mutations may lead to skeletal or cardiomyopathies.

Currently, there are numerous myopathies associated with mutations in myosin-encoding genes, however these mutations are usually dominant and present due to accumulation of myosin in the muscle (Laing and Nowak, 2005). Loss of function *UNC45B* mutations may result in similar phenotypes but would likely follow a recessive pattern of inheritance. Alternatively it is possible that mutations that increase the expression or the stability of *UNC45B* would have deleterious effects on human health. In humans, mutations in *p97* produce inclusion-body myopathy (Watts et al., 2004). This chaperone has been shown to be involved in the maintenance of proper UNC-45 levels and, in cell culture, the myopathy-associated mutations result in detrimental stabilization of human *UNC45B* and loss of muscle organization, possibly directly linked to the altered *UNC45B* levels. This strongly suggests then that any mutation that interferes with *UNC45B* function *or* expression levels would likely adversely affect human muscle formation and function. Therefore it may be worthwhile investigating whether *UNC45B* is affected in patients with currently unmapped myopathies.

4.8 Conclusions

In conclusion, I demonstrate here that there exist two UNC-45 homologues in mammals and zebrafish indicating that this gene has been duplicated following the divergence of vertebrates and invertebrates but that it has not been further duplicated in zebrafish. In zebrafish *unc45a* and *unc45b* are expressed in distinct tissues and disruption of their respective functions results in vastly different phenotypes. *Unc45b* is shown to be involved in the proper development of sarcomeres, downstream of muscle

differentiation in vertebrates. In the absence of Unc45b in zebrafish, embryos are paralyzed and display cardiac dysfunction due to a reduction in functional myosin, consistent with the proposed role for Unc45b as a myosin chaperone or co-chaperone. This suggests that the role of UNC-45 in *C. elegans* is conserved throughout evolution. The results described here provide an important rationale for investigating the roles of specific myosin heavy chain isoforms and the process of their maturation in vertebrate development.

5. Footnotes

Excerpts of this thesis have been published.

Wohlgemuth, S. L., Crawford, B. D. and Pilgrim, D. B. (2007) The myosin co-chaperone UNC-45 is required for skeletal and cardiac muscle function in zebrafish. *Dev Biol* **303**, 483-92.

6. References

- Agarkova, I., and Perriard, J. C. (2005). The M-band: an elastic web that crosslinks thick filaments in the center of the sarcomere. *Trends Cell Biol* **15**, 477-85.
- Ahlberg, P.E. and Milner, A.R. (1994) The origin and early diversification of tetrapods. *Nature* **368**, 507-514.
- Ahmed, Z. M., Morell, R. J., Riazuddin, S., Gropman, A., Shaukat, S., Ahmad, M. M., Mohiddin, S. A., Fananapazir, L., Caruso, R. C., Husnain, T., Khan, S. N., Riazuddin, S., Griffith, A. J., Friedman, T. B., and Wilcox, E. R. (2003). Mutations of MYO6 are associated with recessive deafness, DFNB37. *Am J Hum Genet* **72**, 1315-22.
- Alexander, J., Stainier, D. Y., and Yelon, D. (1998). Screening mosaic F1 females for mutations affecting zebrafish heart induction and patterning. *Dev Genet* **22**, 288-99.
- Amores, A., Force, A., Yan, Y. L., Joly, L., Amemiya, C., Fritz, A., Ho, R. K., Langeland, J., Prince, V., Wang, Y. L., Westerfield, M., Ekker, M., and Postlethwait, J. H. (1998). Zebrafish hox clusters and vertebrate genome evolution. *Science* **282**, 1711-4.
- Amsterdam, A., and Hopkins, N. (2006). Mutagenesis strategies in zebrafish for identifying genes involved in development and disease. *Trends Genet* **22**, 473-8.
- Ao, W. (2001) Ph.D. Thesis. *Caenorhabditis elegans* UNC-45 defines a novel class of myosin-associated proteins. Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada
- Ao, W., and Pilgrim, D. (2000). *Caenorhabditis elegans* UNC-45 is a component of muscle thick filaments and colocalizes with myosin heavy chain B, but not myosin heavy chain A. *J Cell Biol* **148**, 375-84.
- Bader, D., Masaki, T., and Fischman, D. A. (1982). Immunochemical analysis of myosin heavy chain during avian myogenesis in vivo and in vitro. *J Cell Biol* **95**, 763-70.
- Bahary, N., Davidson, A., Ransom, D., Shepard, J., Stern, H., Trede, N., Zhou, Y., Barut, B., and Zon, L. I. (2004). The Zon laboratory guide to positional cloning in zebrafish. *Methods Cell Biol* **77**, 305-29.
- Baker, J. P., and Titus, M. A. (1997). A family of unconventional myosins from the nematode *Caenorhabditis elegans*. *J Mol Biol* **272**, 523-35.

- Barohn, R. J., Brumback, R. A., and Mendell, J. R. (1994). Hyaline body myopathy. *Neuromuscul Disord* **4**, 257-62.
- Barral, J. M., Bauer, C. C., Ortiz, I., and Epstein, H. F. (1998). Unc-45 mutations in *Caenorhabditis elegans* implicate a CRO1/She4p-like domain in myosin assembly. *J Cell Biol* **143**, 1215-25.
- Barral, J. M., Broadley, S. A., Schaffar, G., and Hartl, F. U. (2004). Roles of molecular chaperones in protein misfolding diseases. *Semin Cell Dev Biol* **15**, 17-29.
- Barral, J. M., Hutagalung, A. H., Brinker, A., Hartl, F. U., and Epstein, H. F. (2002). Role of the myosin assembly protein UNC-45 as a molecular chaperone for myosin. *Science* **295**, 669-71.
- Bartman, T., Walsh, E. C., Wen, K. K., McKane, M., Ren, J., Alexander, J., Rubenstein, P. A., and Stainier, D. Y. (2004). Early myocardial function affects endocardial cushion development in zebrafish. *PLoS Biol* **2**, E129.
- Bartram, C., Edwards, R. H., and Beynon, R. J. (1995). McArdle's disease-muscle glycogen phosphorylase deficiency. *Biochim Biophys Acta* **1272**, 1-13.
- Bashyam, M. D., Savithri, G. R., Kumar, M. S., Narasimhan, C., and Nallari, P. (2003). Molecular genetics of familial hypertrophic cardiomyopathy (FHC). *J Hum Genet* **48**, 55-64.
- Berdougo, E., Coleman, H., Lee, D. H., Stainier, D. Y., and Yelon, D. (2003). Mutation of weak atrium/atrial myosin heavy chain disrupts atrial function and influences ventricular morphogenesis in zebrafish. *Development* **130**, 6121-9.
- Berg, J. S., Powell, B. C., and Cheney, R. E. (2001). A millennial myosin census. *Mol Biol Cell* **12**, 780-94.
- Blair, E., Redwood, C., de Jesus Oliveira, M., Moolman-Smook, J. C., Brink, P., Corfield, V. A., Ostman-Smith, I., and Watkins, H. (2002). Mutations of the light meromyosin domain of the beta-myosin heavy chain rod in hypertrophic cardiomyopathy. *Circ Res* **90**, 263-9.
- Bobola, N., Jansen, R. P., Shin, T. H., and Nasmyth, K. (1996). Asymmetric accumulation of Ash1p in postanaphase nuclei depends on a myosin and restricts yeast mating-type switching to mother cells. *Cell* **84**, 699-709.
- Bonnemann, C. G., and Laing, N. G. (2004). Myopathies resulting from mutations in sarcomeric proteins. *Curr Opin Neurol* **17**, 529-37.

- Brinker, A., Scheufler, C., Von Der Mulbe, F., Fleckenstein, B., Herrmann, C., Jung, G., Moarefi, I., and Hartl, F. U. (2002). Ligand discrimination by TPR domains. Relevance and selectivity of EEVD-recognition in Hsp70 x Hop x Hsp90 complexes. *J Biol Chem* **277**, 19265-75.
- Bukau, B., Weissman, J., and Horwich, A. (2006). Molecular chaperones and protein quality control. *Cell* **125**, 443-51.
- Callen, J. P., and Wortmann, R. L. (2006). Dermatomyositis. *Clin Dermatol* **24**, 363-73.
- Caplan, A. J. (2003). What is a co-chaperone? *Cell Stress Chaperones* **8**, 105-7.
- Chadli, A., Graham, J. D., Abel, M. G., Jackson, T. A., Gordon, D. F., Wood, W. M., Felts, S. J., Horwitz, K. B., and Toft, D. (2006). GCUNC-45 is a novel regulator for the progesterone receptor/hsp90 chaperoning pathway. *Mol Cell Biol* **26**, 1722-30.
- Chang, A. N., and Potter, J. D. (2005). Sarcomeric protein mutations in dilated cardiomyopathy. *Heart Fail Rev* **10**, 225-35.
- Checkland, T. (2004) M.Sc. Thesis. A *Drosophila* member of the UCS protein family. Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada
- Chen, J. N., Haffter, P., Odenthal, J., Vogelsang, E., Brand, M., van Eeden, F. J., Furutani-Seiki, M., Granato, M., Hammerschmidt, M., Heisenberg, C. P., Jiang, Y. J., Kane, D. A., Kelsh, R. N., Mullins, M. C., and Nusslein-Volhard, C. (1996). Mutations affecting the cardiovascular system and other internal organs in zebrafish. *Development* **123**, 293-302.
- Chow, D., Srikakulam, R., Chen, Y., and Winkelmann, D. A. (2002). Folding of the striated muscle myosin motor domain. *J Biol Chem* **277**, 36799-807.
- Ciruna, B., Weidinger, G., Knaut, H., Thisse, B., Thisse, C., Raz, E., and Schier, A. F. (2002). Production of maternal-zygotic mutant zebrafish by germ-line replacement. *Proc Natl Acad Sci U S A* **99**, 14919-24.
- Clark, K. A., McElhinny, A. S., Beckerle, M. C., and Gregorio, C. C. (2002). Striated muscle cytoarchitecture: an intricate web of form and function. *Annu Rev Cell Dev Biol* **18**, 637-706.
- Connell, P., Ballinger, C. A., Jiang, J., Wu, Y., Thompson, L. J., Hohfeld, J., and Patterson, C. (2001). The co-chaperone CHIP regulates protein triage decisions mediated by heat-shock proteins. *Nat Cell Biol* **3**, 93-6.

- Coutelle, O., Blagden, C. S., Hampson, R., Halai, C., Rigby, P. W., and Hughes, S. M. (2001). Hedgehog signalling is required for maintenance of myf5 and myoD expression and timely terminal differentiation in zebrafish adaxial myogenesis. *Dev Biol* **236**, 136-50.
- Craig, R., and Woodhead, J. L. (2006). Structure and function of myosin filaments. *Curr Opin Struct Biol* **16**, 204-12.
- Crow, M. T., and Stockdale, F. E. (1986). Myosin expression and specialization among the earliest muscle fibers of the developing avian limb. *Dev Biol* **113**, 238-54.
- Dalakas, M. C. (2006). Mechanisms of disease: signaling pathways and immunobiology of inflammatory myopathies. *Nat Clin Pract Rheumatol* **2**, 219-27.
- Dalakas, M. C., and Hohlfeld, R. (2003). Polymyositis and dermatomyositis. *Lancet* **362**, 971-82.
- Darin, N., Tajsharghi, H., Ostman-Smith, I., Gilljam, T., and Oldfors, A. (2007). New skeletal myopathy and cardiomyopathy associated with a missense mutation in MYH7. *Neurology* **68**, 2041-2.
- Devoto, S. H., Melancon, E., Eisen, J. S., and Westerfield, M. (1996). Identification of separate slow and fast muscle precursor cells in vivo, prior to somite formation. *Development* **122**, 3371-80.
- DiMauro, S. (2006). Mitochondrial myopathies. *Curr Opin Rheumatol* **18**, 636-41.
- DiMauro, S., and Gurgel-Giannetti, J. (2005). The expanding phenotype of mitochondrial myopathy. *Curr Opin Neurol* **18**, 538-42.
- Drabikowski, W., Barylko, B., Dabrowska, R., and Nowak, E. (1968). Binding of calcium by troponin. *Bull Acad Pol Sci Biol* **16**, 397-400.
- Draper, B. W., McCallum, C. M., Stout, J. L., Slade, A. J., and Moens, C. B. (2004). A high-throughput method for identifying N-ethyl-N-nitrosourea (ENU)-induced point mutations in zebrafish. *Methods Cell Biol* **77**, 91-112.
- Draper, B. W., Stock, D. W., and Kimmel, C. B. (2003). Zebrafish fgf24 functions with fgf8 to promote posterior mesodermal development. *Development* **130**, 4639-54.
- Ebert, A. M., Hume, G. L., Warren, K. S., Cook, N. P., Burns, C. G., Mohideen, M. A., Siegal, G., Yelon, D., Fishman, M. C., and Garrity, D. M. (2005). Calcium extrusion is critical for cardiac morphogenesis and rhythm in embryonic zebrafish hearts. *Proc Natl Acad Sci U S A* **102**, 17705-10.
- Epstein, H. F., and Thomson, J. N. (1974). Temperature-sensitive mutation affecting myofilament assembly in *Caenorhabditis elegans*. *Nature* **250**, 579-80.

- Ernest, S., Rauch, G. J., Haffter, P., Geisler, R., Petit, C., and Nicolson, T. (2000). Mariner is defective in myosin VIIA: a zebrafish model for human hereditary deafness. *Hum Mol Genet* **9**, 2189-96.
- Etard, C., Behra, M., Fischer, N., Hutcheson, D., Geisler, R., and Strahle, U. (2007). The UCS factor Steif/Unc-45b interacts with the heat shock protein Hsp90a during myofibrillogenesis. *Dev Biol* **308**, 133-43.
- Etheridge, L., Diiorio, P., and Sagerstrom, C. G. (2002). A zebrafish unc-45-related gene expressed during muscle development. *Dev Dyn* **224**, 457-60.
- Fananapazir, L., Dalakas, M. C., Cyran, F., Cohn, G., and Epstein, N. D. (1993). Missense mutations in the beta-myosin heavy-chain gene cause central core disease in hypertrophic cardiomyopathy. *Proc Natl Acad Sci U S A* **90**, 3993-7.
- Foth, B. J., Goedecke, M. C., and Soldati, D. (2006). New insights into myosin evolution and classification. *Proc Natl Acad Sci U S A* **103**, 3681-6.
- Fox, S. I. (2004) Muscle. In *Human Physiology*, ed. M. Watnick, K. A. Queck, pp. 324-363. New York, NY, USA; McGraw-Hill
- Fukuda, T., Roberts, A., Plotz, P. H., and Raben, N. (2007). Acid alpha-glucosidase deficiency (Pompe disease). *Curr Neurol Neurosci Rep* **7**, 71-7.
- Garrity, D. M., Childs, S., and Fishman, M. C. (2002). The heartstrings mutation in zebrafish causes heart/fin Tbx5 deficiency syndrome. *Development* **129**, 4635-45.
- Gerull, B., Gramlich, M., Atherton, J., McNabb, M., Trombitas, K., Sasse-Klaassen, S., Seidman, J. G., Seidman, C., Granzier, H., Labeit, S., Frenneaux, M., and Thierfelder, L. (2002). Mutations of TTN, encoding the giant muscle filament titin, cause familial dilated cardiomyopathy. *Nat Genet* **30**, 201-4.
- Gleason, M. R., Armisen, R., Verdecia, M. A., Sirotkin, H., Brehm, P., and Mandel, G. (2004). A mutation in serca underlies motility dysfunction in accordion zebrafish. *Dev Biol* **276**, 441-51.
- Gottlieb, P. D., Pierce, S. A., Sims, R. J., Yamagishi, H., Weihe, E. K., Harriss, J. V., Maika, S. D., Kuziel, W. A., King, H. L., Olson, E. N., Nakagawa, O., and Srivastava, D. (2002). Bop encodes a muscle-restricted protein containing MYND and SET domains and is essential for cardiac differentiation and morphogenesis. *Nat Genet* **31**, 25-32.

- Granato, M., van Eeden, F. J., Schach, U., Trowe, T., Brand, M., Furutani-Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C. P., Jiang, Y. J., Kane, D. A., Kelsh, R. N., Mullins, M. C., Odenthal, J., and Nusslein-Volhard, C. (1996). Genes controlling and mediating locomotion behavior of the zebrafish embryo and larva. *Development* **123**, 399-413.
- Granger, B. L., and Lazarides, E. (1979). Desmin and vimentin coexist at the periphery of the myofibril Z disc. *Cell* **18**, 1053-63.
- Granzier, H. L., and Campbell, K. B. (2006). New insights in the role of cardiac myosin binding protein C as a regulator of cardiac contractility. *Circ Res* **99**, 795-7.
- Gregersen, N. (2006). Protein misfolding disorders: pathogenesis and intervention. *J Inherit Metab Dis* **29**, 456-70.
- Guo, S., and Kemphues, K. J. (1996). A non-muscle myosin required for embryonic polarity in *Caenorhabditis elegans*. *Nature* **382**, 455-8.
- Hamade, A., Deries, M., Begemann, G., Bally-Cuif, L., Genet, C., Sabatier, F., Bonniieu, A., and Cousin, X. (2006). Retinoic acid activates myogenesis in vivo through Fgf8 signalling. *Dev Biol* **289**, 127-40.
- Hasson, T. (2003). Myosin VI: two distinct roles in endocytosis. *J Cell Sci* **116**, 3453-61.
- Heckmann, J. G., Dietrich, W., Hohenberger, W., Klein, P., Hanke, B., and Neundorfer, B. (2000). Hypoglycemic sensorimotor polyneuropathy associated with insulinoma. *Muscle Nerve* **23**, 1891-4.
- Hekimsoy, Z., and Oktem, I. K. (2005). Serum creatine kinase levels in overt and subclinical hypothyroidism. *Endocr Res* **31**, 171-5.
- Hernandez, O. M., Jones, M., Guzman, G., and Szczesna-Cordary, D. (2007). Myosin essential light chain in health and disease. *Am J Physiol Heart Circ Physiol* **292**, H1643-54.
- Homma, K., Yoshimura, M., Saito, J., Ikebe, R., and Ikebe, M. (2001). The core of the motor domain determines the direction of myosin movement. *Nature* **412**, 831-4.
- Hoppe, P. E., Andrews, R. C., and Parikh, P. D. (2003). Differential requirement for the nonhelical tailpiece and the C terminus of the myosin rod in *Caenorhabditis elegans* muscle. *Mol Biol Cell* **14**, 1677-90.
- Hoppe, T., Cassata, G., Barral, J. M., Springer, W., Hutagalung, A. H., Epstein, H. F., and Baumeister, R. (2004). Regulation of the myosin-directed chaperone UNC-45 by a novel E3/E4-multiubiquitylation complex in *C. elegans*. *Cell* **118**, 337-49.

- Horlick, L. (2005) M.Sc. Thesis. Molecular interactions between MHC B and its co-chaperone, UNC-45 in *C. elegans*. Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada
- Horowitz, R., Kempner, E. S., Bisher, M. E., and Podolsky, R. J. (1986). A physiological role for titin and nebulin in skeletal muscle. *Nature* **323**, 160-4.
- Hove, J. R., Koster, R. W., Forouhar, A. S., Acevedo-Bolton, G., Fraser, S. E., and Gharib, M. (2003). Intracardiac fluid forces are an essential epigenetic factor for embryonic cardiogenesis. *Nature* **421**, 172-7.
- Hutagalung, A. H., Landsverk, M. L., Price, M. G., and Epstein, H. F. (2002). The UCS family of myosin chaperones. *J Cell Sci* **115**, 3983-90.
- Huxley, H. E., and Brown, W. (1967). The low-angle x-ray diagram of vertebrate striated muscle and its behaviour during contraction and rigor. *J Mol Biol* **30**, 383-434.
- Huxley, H.E. and Hanson J., Changes in the cross striations of muscle during contraction and stretch and their structural interpretation. *Nature* **173** (1954), 973-976.
- Imai, Y., Soda, M., Hatakeyama, S., Akagi, T., Hashikawa, T., Nakayama, K. I., and Takahashi, R. (2002). CHIP is associated with Parkin, a gene responsible for familial Parkinson's disease, and enhances its ubiquitin ligase activity. *Mol Cell* **10**, 55-67.
- Jackson, C. E., and Bryan, W. W. (1998). Amyotrophic lateral sclerosis. *Semin Neurol* **18**, 27-39.
- Jandreski, M. A., Sole, M. J., and Liew, C. C. (1987). Two different forms of beta myosin heavy chain are expressed in human striated muscle. *Hum Genet* **77**, 127-31.
- Janiesch, P. C., Kim, J., Mouysset, J., Barikbin, R., Lochmuller, H., Cassata, G., Krause, S., and Hoppe, T. (2007). The ubiquitin-selective chaperone CDC-48/p97 links myosin assembly to human myopathy. *Nat Cell Biol* **9**, 379-90.
- Jansen, R. P., Dowzer, C., Michaelis, C., Galova, M., and Nasmyth, K. (1996). Mother cell-specific HO expression in budding yeast depends on the unconventional myosin myo4p and other cytoplasmic proteins. *Cell* **84**, 687-97.
- Kachur, T., Ao, W., Berger, J., and Pilgrim, D. (2004). Maternal UNC-45 is involved in cytokinesis and colocalizes with non-muscle myosin in the early *Caenorhabditis elegans* embryo. *J Cell Sci* **117**, 5313-21.
- Kachur, T.M., Audhya, A., and Pilgrim, D.B. (2007) UNC-45 is required for NMY-2 contractile function in early embryonic polarity establishment and germline cellularization in *C. elegans* (submitted)

- Kappler, J. A., Starr, C. J., Chan, D. K., Kollmar, R., and Hudspeth, A. J. (2004). A nonsense mutation in the gene encoding a zebrafish myosin VI isoform causes defects in hair-cell mechanotransduction. *Proc Natl Acad Sci U S A* **101**, 13056-61.
- Karlstrom, R. O., Trowe, T., Klostermann, S., Baier, H., Brand, M., Crawford, A. D., Grunewald, B., Haffter, P., Hoffmann, H., Meyer, S. U., Muller, B. K., Richter, S., van Eeden, F. J., Nusslein-Volhard, C., and Bonhoeffer, F. (1996). Zebrafish mutations affecting retinotectal axon pathfinding. *Development* **123**, 427-38.
- Kerscher, O., Felberbaum, R., and Hochstrasser, M. (2006). Modification of proteins by ubiquitin and ubiquitin-like proteins. *Annu Rev Cell Dev Biol* **22**, 159-80.
- Knappeis, G. G., and Carlsen, F. (1968). The ultrastructure of the M line in skeletal muscle. *J Cell Biol* **38**, 202-11.
- Labeit, S., Barlow, D. P., Gautel, M., Gibson, T., Holt, J., Hsieh, C. L., Francke, U., Leonard, K., Wardale, J., Whiting, A., and et al. (1990). A regular pattern of two types of 100-residue motif in the sequence of titin. *Nature* **345**, 273-6.
- Laing, N. G., and Nowak, K. J. (2005). When contractile proteins go bad: the sarcomere and skeletal muscle disease. *Bioessays* **27**, 809-22.
- Landsverk, M. L., Li, S., Hutagalung, A. H., Najafov, A., Hoppe, T., Barral, J. M., and Epstein, H. F. (2007). The UNC-45 chaperone mediates sarcomere assembly through myosin degradation in *Caenorhabditis elegans*. *J Cell Biol* **177**, 205-10.
- Lister, J. A., Robertson, C. P., Lepage, T., Johnson, S. L., and Raible, D. W. (1999). nacre encodes a zebrafish microphthalmia-related protein that regulates neural-crest-derived pigment cell fate. *Development* **126**, 3757-67.
- Liu, X. Z., Walsh, J., Mburu, P., Kendrick-Jones, J., Cope, M. J., Steel, K. P., and Brown, S. D. (1997a). Mutations in the myosin VIIA gene cause non-syndromic recessive deafness. *Nat Genet* **16**, 188-90.
- Liu, X. Z., Walsh, J., Tamagawa, Y., Kitamura, K., Nishizawa, M., Steel, K. P., and Brown, S. D. (1997b). Autosomal dominant non-syndromic deafness caused by a mutation in the myosin VIIA gene. *Nat Genet* **17**, 268-9.
- Lord, M., and Pollard, T. D. (2004). UCS protein Rng3p activates actin filament gliding by fission yeast myosin-II. *J Cell Biol* **167**, 315-25.
- Lowey, S., Waller, G. S., and Trybus, K. M. (1993). Skeletal muscle myosin light chains are essential for physiological speeds of shortening. *Nature* **365**, 454-6.

- Lundberg, I. E., and Alexanderson, H. (2007). Technology insight: tools for research, diagnosis and clinical assessment of treatment in idiopathic inflammatory myopathies. *Nat Clin Pract Rheumatol* **3**, 282-90.
- Macario, A. J., and Conway de Macario, E. (2005). Sick chaperones, cellular stress, and disease. *N Engl J Med* **353**, 1489-501.
- Maceluch, J. A., and Niedziela, M. (2006). The clinical diagnosis and molecular genetics of kearns-sayre syndrome: a complex mitochondrial encephalomyopathy. *Pediatr Endocrinol Rev* **4**, 117-37.
- Mayer, M. P., and Bukau, B. (2005). Hsp70 chaperones: cellular functions and molecular mechanism. *Cell Mol Life Sci* **62**, 670-84.
- McElhinny, A. S., Schwach, C., Valichnac, M., Mount-Patrick, S., and Gregorio, C. C. (2005). Nebulin regulates the assembly and lengths of the thin filaments in striated muscle. *J Cell Biol* **170**, 947-57.
- McLachlan, A. D., and Karn, J. (1983). Periodic features in the amino acid sequence of nematode myosin rod. *J Mol Biol* **164**, 605-26.
- Melchionda, S., Ahituv, N., Bisceglia, L., Sobe, T., Glaser, F., Rabionet, R., Arbones, M. L., Notarangelo, A., Di Iorio, E., Carella, M., Zelante, L., Estivill, X., Avraham, K. B., and Gasparini, P. (2001). MYO6, the human homologue of the gene responsible for deafness in Snell's waltzer mice, is mutated in autosomal dominant nonsyndromic hearing loss. *Am J Hum Genet* **69**, 635-40.
- Meredith, C., Herrmann, R., Parry, C., Liyanage, K., Dye, D. E., Durling, H. J., Duff, R. M., Beckman, K., de Visser, M., van der Graaff, M. M., Hedera, P., Fink, J. K., Petty, E. M., Lamont, P., Fabian, V., Bridges, L., Voit, T., Mastaglia, F. L., and Laing, N. G. (2004). Mutations in the slow skeletal muscle fiber myosin heavy chain gene (MYH7) cause laing early-onset distal myopathy (MPD1). *Am J Hum Genet* **75**, 703-8.
- Miller, D. M., 3rd, Ortiz, I., Berliner, G. C., and Epstein, H. F. (1983). Differential localization of two myosins within nematode thick filaments. *Cell* **34**, 477-90.
- Mishra, M., D'Souza V, M., Chang, K. C., Huang, Y., and Balasubramanian, M. K. (2005). Hsp90 protein in fission yeast Swolp and UCS protein Rng3p facilitate myosin II assembly and function. *Eukaryot Cell* **4**, 567-76.
- Morita, H., Seidman, J., and Seidman, C. E. (2005). Genetic causes of human heart failure. *J Clin Invest* **115**, 518-26.

- Murakami, K., Yumoto, F., Ohki, S. Y., Yasunaga, T., Tanokura, M., and Wakabayashi, T. (2007). Structural basis for calcium-regulated relaxation of striated muscles at interaction sites of troponin with actin and tropomyosin. *Adv Exp Med Biol* **592**, 71-86.
- Nakasawa, T., Takahashi, M., Matsuzawa, F., Aikawa, S., Togashi, Y., Saitoh, T., Yamagishi, A., and Yazawa, M. (2005). Critical regions for assembly of vertebrate nonmuscle myosin II. *Biochemistry* **44**, 174-83.
- Nasevicius, A., and Ekker, S. C. (2000). Effective targeted gene 'knockdown' in zebrafish. *Nat Genet* **26**, 216-20.
- Nathan, D. F., Vos, M. H., and Lindquist, S. (1997). In vivo functions of the *Saccharomyces cerevisiae* Hsp90 chaperone. *Proc Natl Acad Sci U S A* **94**, 12949-56.
- Neuhauss, S. C., Solnica-Krezel, L., Schier, A. F., Zwartkruis, F., Stemple, D. L., Malicki, J., Abdelilah, S., Stainier, D. Y., and Driever, W. (1996). Mutations affecting craniofacial development in zebrafish. *Development* **123**, 357-67.
- Nicolson, T., Rusch, A., Friedrich, R. W., Granato, M., Ruppertsberg, J. P., and Nusslein-Volhard, C. (1998). Genetic analysis of vertebrate sensory hair cell mechanosensation: the zebrafish circler mutants. *Neuron* **20**, 271-83.
- Nishikawa, S., Homma, K., Komori, Y., Iwaki, M., Wazawa, T., Hikikoshi Iwane, A., Saito, J., Ikebe, R., Katayama, E., Yanagida, T., and Ikebe, M. (2002). Class VI myosin moves processively along actin filaments backward with large steps. *Biochem Biophys Res Commun* **290**, 311-7.
- Ochi, H., Pearson, B. J., Chuang, P. T., Hammerschmidt, M., and Westerfield, M. (2006). Hhip regulates zebrafish muscle development by both sequestering Hedgehog and modulating localization of Smoothened. *Dev Biol* **297**, 127-40.
- Ochi, H., and Westerfield, M. (2007). Signaling networks that regulate muscle development: lessons from zebrafish. *Dev Growth Differ* **49**, 1-11.
- Pan, Y., Zvaritch, E., Tupling, A. R., Rice, W. J., de Leon, S., Rudnicki, M., McKerlie, C., Banwell, B. L., and MacLennan, D. H. (2003). Targeted disruption of the ATP2A1 gene encoding the sarco(endo)plasmic reticulum Ca²⁺ ATPase isoform 1 (SERCA1) impairs diaphragm function and is lethal in neonatal mice. *J Biol Chem* **278**, 13367-75.
- Parsons, M. J., Campos, I., Hirst, E. M., and Stemple, D. L. (2002). Removal of dystroglycan causes severe muscular dystrophy in zebrafish embryos. *Development* **129**, 3505-12.

- Peachey, L. D. (1965). Transverse tubules in excitation-contraction coupling. *Fed Proc* **24**, 1124-34.
- Pearl, L. H., and Prodromou, C. (2006). Structure and mechanism of the Hsp90 molecular chaperone machinery. *Annu Rev Biochem* **75**, 271-94.
- Perry, S. V. (2001). Vertebrate tropomyosin: distribution, properties and function. *J Muscle Res Cell Motil* **22**, 5-49.
- Phenotype Annotation (1994-2006) (2006) Mutant Data Curated from Older Literature. ZFIN Historical Data
- Picard, D. (2002). Heat-shock protein 90, a chaperone for folding and regulation. *Cell Mol Life Sci* **59**, 1640-8.
- Pickart, C. M. (2004). Back to the future with ubiquitin. *Cell* **116**, 181-90.
- Pownall, M. E., Gustafsson, M. K., and Emerson, C. P., Jr. (2002). Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos. *Annu Rev Cell Dev Biol* **18**, 747-83.
- Price, M. G., Landsverk, M. L., Barral, J. M., and Epstein, H. F. (2002). Two mammalian UNC-45 isoforms are related to distinct cytoskeletal and muscle-specific functions. *J Cell Sci* **115**, 4013-23.
- Raeker, M. O., Su, F., Geisler, S. B., Borisov, A. B., Kontogianni-Konstantopoulos, A., Lyons, S. E., and Russell, M. W. (2006). Obscurin is required for the lateral alignment of striated myofibrils in zebrafish. *Dev Dyn* **235**, 2018-29.
- Rauch, G.J., Lyons, D.A., Middendorf, I., Friedlander, B., Arana, N., Reyes, T., and Talbot, W.S. (2003) Submission and Curation of Gene Expression Data. ZFIN Direct Data Submission
- Redwood, C. S., Moolman-Smook, J. C., and Watkins, H. (1999). Properties of mutant contractile proteins that cause hypertrophic cardiomyopathy. *Cardiovasc Res* **44**, 20-36.
- Rudnicki, M. A., Schnegelsberg, P. N., Stead, R. H., Braun, T., Arnold, H. H., and Jaenisch, R. (1993). MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell* **75**, 1351-9.
- Sass, J. B., Martin, C. C., and Krone, P. H. (1999). Restricted expression of the zebrafish hsp90alpha gene in slow and fast muscle fiber lineages. *Int J Dev Biol* **43**, 835-8.

- Sass, J. B., Weinberg, E. S., and Krone, P. H. (1996). Specific localization of zebrafish hsp90 alpha mRNA to myoD-expressing cells suggests a role for hsp90 alpha during normal muscle development. *Mech Dev* **54**, 195-204.
- Scheufler, C., Brinker, A., Bourenkov, G., Pegoraro, S., Moroder, L., Bartunik, H., Hartl, F. U., and Moarefi, I. (2000). Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. *Cell* **101**, 199-210.
- Schilling, T. F., and Kimmel, C. B. (1997). Musculoskeletal patterning in the pharyngeal segments of the zebrafish embryo. *Development* **124**, 2945-60.
- Schilling, T. F., Piotrowski, T., Grandel, H., Brand, M., Heisenberg, C. P., Jiang, Y. J., Beuchle, D., Hammerschmidt, M., Kane, D. A., Mullins, M. C., van Eeden, F. J., Kelsh, R. N., Furutani-Seiki, M., Granato, M., Haffter, P., Odenthal, J., Warga, R. M., Trowe, T., and Nusslein-Volhard, C. (1996). Jaw and branchial arch mutants in zebrafish I: branchial arches. *Development* **123**, 329-44.
- Sehnert, A. J., Huq, A., Weinstein, B. M., Walker, C., Fishman, M., and Stainier, D. Y. (2002). Cardiac troponin T is essential in sarcomere assembly and cardiac contractility. *Nat Genet* **31**, 106-10.
- Seiler, C., Ben-David, O., Sidi, S., Hendrich, O., Rusch, A., Burnside, B., Avraham, K. B., and Nicolson, T. (2004). Myosin VI is required for structural integrity of the apical surface of sensory hair cells in zebrafish. *Dev Biol* **272**, 328-38.
- Serluca, F. C., Drummond, I. A., and Fishman, M. C. (2002). Endothelial signaling in kidney morphogenesis: a role for hemodynamic forces. *Curr Biol* **12**, 492-7.
- Shelton, C. A., Carter, J. C., Ellis, G. C., and Bowerman, B. (1999). The nonmuscle myosin regulatory light chain gene *mlc-4* is required for cytokinesis, anterior-posterior polarity, and body morphology during *Caenorhabditis elegans* embryogenesis. *J Cell Biol* **146**, 439-51.
- Sherwood, L. (2001) Muscle Physiology. In *Human Physiology From Cells to Systems*, ed. N. Horne, K. Milotich, M. Arbogast, K. Bomont, M. Pinette, pp. 239-281. Pacific Grove, CA, USA; Brooks/Cole
- Shoesmith, C. L., and Strong, M. J. (2006). Amyotrophic lateral sclerosis: update for family physicians. *Can Fam Physician* **52**, 1563-9.
- Smith, D. F., Schowalter, D. B., Kost, S. L., and Toft, D. O. (1990). Reconstitution of progesterone receptor with heat shock proteins. *Mol Endocrinol* **4**, 1704-11.
- Srikakulam, R., and Winkelmann, D. A. (1999). Myosin II folding is mediated by a molecular chaperonin. *J Biol Chem* **274**, 27265-73.

- Srikakulam, R., and Winkelmann, D. A. (2004). Chaperone-mediated folding and assembly of myosin in striated muscle. *J Cell Sci* **117**, 641-52.
- Stainier, D. Y. (2001). Zebrafish genetics and vertebrate heart formation. *Nat Rev Genet* **2**, 39-48.
- Stainier, D. Y., and Fishman, M. C. (1992). Patterning the zebrafish heart tube: acquisition of anteroposterior polarity. *Dev Biol* **153**, 91-101.
- Stainier, D. Y., Fouquet, B., Chen, J. N., Warren, K. S., Weinstein, B. M., Meiler, S. E., Mohideen, M. A., Neuhauss, S. C., Solnica-Krezel, L., Schier, A. F., Zwartkruis, F., Stemple, D. L., Malicki, J., Driever, W., and Fishman, M. C. (1996). Mutations affecting the formation and function of the cardiovascular system in the zebrafish embryo. *Development* **123**, 285-92.
- Stemple, D. L. (2004). TILLING--a high-throughput harvest for functional genomics. *Nat Rev Genet* **5**, 145-50.
- Stepkowski, D. (1995). The role of the skeletal muscle myosin light chains N-terminal fragments. *FEBS Lett* **374**, 6-11.
- Stewart, M., and Kensler, R. W. (1986). Arrangement of myosin heads in relaxed thick filaments from frog skeletal muscle. *J Mol Biol* **192**, 831-51.
- Stromer, M. H. (1995). Immunocytochemistry of the muscle cell cytoskeleton. *Microsc Res Tech* **31**, 95-105.
- Sung, S. S., Brassington, A. M., Grannatt, K., Rutherford, A., Whitby, F. G., Krakowiak, P. A., Jorde, L. B., Carey, J. C., and Bamshad, M. (2003a). Mutations in genes encoding fast-twitch contractile proteins cause distal arthrogryposis syndromes. *Am J Hum Genet* **72**, 681-90.
- Sung, S. S., Brassington, A. M., Krakowiak, P. A., Carey, J. C., Jorde, L. B., and Bamshad, M. (2003b). Mutations in TNNT3 cause multiple congenital contractures: a second locus for distal arthrogryposis type 2B. *Am J Hum Genet* **73**, 212-4.
- Sweeney, H. L., Bowman, B. F., and Stull, J. T. (1993). Myosin light chain phosphorylation in vertebrate striated muscle: regulation and function. *Am J Physiol* **264**, C1085-95.
- Szczesna, D. (2003). Regulatory light chains of striated muscle myosin. Structure, function and malfunction. *Curr Drug Targets Cardiovasc Haematol Disord* **3**, 187-97.

- Szeto, D. P., Griffin, K. J., and Kimelman, D. (2002). HrT is required for cardiovascular development in zebrafish. *Development* **129**, 5093-101.
- Tan, X., Rotllant, J., Li, H., De Deyne, P., and Du, S. J. (2006). SmyD1, a histone methyltransferase, is required for myofibril organization and muscle contraction in zebrafish embryos. *Proc Natl Acad Sci U S A* **103**, 2713-8.
- Tardiff, J. C. (2005). Sarcomeric proteins and familial hypertrophic cardiomyopathy: linking mutations in structural proteins to complex cardiovascular phenotypes. *Heart Fail Rev* **10**, 237-48.
- Taylor, J. S., Braasch, I., Frickey, T., Meyer, A., and Van de Peer, Y. (2003). Genome duplication, a trait shared by 22000 species of ray-finned fish. *Genome Res* **13**, 382-90.
- Thierfelder, L., Watkins, H., MacRae, C., Lamas, R., McKenna, W., Vosberg, H. P., Seidman, J. G., and Seidman, C. E. (1994). Alpha-tropomyosin and cardiac troponin T mutations cause familial hypertrophic cardiomyopathy: a disease of the sarcomere. *Cell* **77**, 701-12.
- Thisse, B., Pflumio, S., Fürthauer, M., Loppin, B., Heyer, V., Degraeve, A., Woehl, R., Lux, A., Steffan, T., Charbonnier, X.Q. and Thisse, C. (2001) Expression of the zebrafish genome during embryogenesis (NIH R01 RR15402). ZFIN Direct Data Submission
- Thisse, B., Thisse, C. (2004) Fast Release Clones: A High Throughput Expression Analysis. ZFIN Direct Data Submission
- Thisse, C., and Thisse, B. (2005) High Throughput Expression Analysis of ZF-Models Consortium Clones. ZFIN Direct Data Submission
- Timson, D. J. (2003). Fine tuning the myosin motor: the role of the essential light chain in striated muscle myosin. *Biochimie* **85**, 639-45.
- Toi, H., Fujimura-Kamada, K., Irie, K., Takai, Y., Todo, S., and Tanaka, K. (2003). She4p/Dim1p interacts with the motor domain of unconventional myosins in the budding yeast, *Saccharomyces cerevisiae*. *Mol Biol Cell* **14**, 2237-49.
- Tskhovrebova, L., and Trinick, J. (2003). Titin: properties and family relationships. *Nat Rev Mol Cell Biol* **4**, 679-89.
- Tyska, M. J., and Warshaw, D. M. (2002). The myosin power stroke. *Cell Motil Cytoskeleton* **51**, 1-15.
- Urven, L. E., Yabe, T., and Pelegri, F. (2006). A role for non-muscle myosin II function in furrow maturation in the early zebrafish embryo. *J Cell Sci* **119**, 4342-52.

- Venolia, L., Ao, W., Kim, S., Kim, C., and Pilgrim, D. (1999). unc-45 gene of *Caenorhabditis elegans* encodes a muscle-specific tetratricopeptide repeat-containing protein. *Cell Motil Cytoskeleton* **42**, 163-77.
- Venolia, L., and Waterston, R. H. (1990). The unc-45 gene of *Caenorhabditis elegans* is an essential muscle-affecting gene with maternal expression. *Genetics* **126**, 345-53.
- Wallace, K. N., Dolan, A. C., Seiler, C., Smith, E. M., Yusuff, S., Chaille-Arnold, L., Judson, B., Sierk, R., Yengo, C., Sweeney, H. L., and Pack, M. (2005). Mutation of smooth muscle myosin causes epithelial invasion and cystic expansion of the zebrafish intestine. *Dev Cell* **8**, 717-26.
- Walton, R. Z., Bruce, A. E., Olivey, H. E., Najib, K., Johnson, V., Earley, J. U., Ho, R. K., and Svensson, E. C. (2006). Fog1 is required for cardiac looping in zebrafish. *Dev Biol* **289**, 482-93.
- Wang, K., McClure, J., and Tu, A. (1979). Titin: major myofibrillar components of striated muscle. *Proc Natl Acad Sci U S A* **76**, 3698-702.
- Wang, K., and Williamson, C. L. (1980). Identification of an N2 line protein of striated muscle. *Proc Natl Acad Sci U S A* **77**, 3254-8.
- Waterston, R. H., Thomson, J. N., and Brenner, S. (1980). Mutants with altered muscle structure of *Caenorhabditis elegans*. *Dev Biol* **77**, 271-302.
- Watts, G. D., Wymer, J., Kovach, M. J., Mehta, S. G., Mumm, S., Darvish, D., Pestronk, A., Whyte, M. P., and Kimonis, V. E. (2004). Inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia is caused by mutant valosin-containing protein. *Nat Genet* **36**, 377-81.
- Weil, D., Blanchard, S., Kaplan, J., Guilford, P., Gibson, F., Walsh, J., Mburu, P., Varela, A., Levilliers, J., Weston, M. D., and et al. (1995). Defective myosin VIIA gene responsible for Usher syndrome type 1B. *Nature* **374**, 60-1.
- Weil, D., Kussel, P., Blanchard, S., Levy, G., Levi-Acobas, F., Drira, M., Ayadi, H., and Petit, C. (1997). The autosomal recessive isolated deafness, DFNB2, and the Usher 1B syndrome are allelic defects of the myosin-VIIA gene. *Nat Genet* **16**, 191-3.
- Weinberg, E. S., Allende, M. L., Kelly, C. S., Abdelhamid, A., Murakami, T., Andermann, P., Doerre, O. G., Grunwald, D. J., and Riggleman, B. (1996). Developmental regulation of zebrafish MyoD in wild-type, no tail and spadetail embryos. *Development* **122**, 271-80.

- Wells, A. L., Lin, A. W., Chen, L. Q., Safer, D., Cain, S. M., Hasson, T., Carragher, B. O., Milligan, R. A., and Sweeney, H. L. (1999). Myosin VI is an actin-based motor that moves backwards. *Nature* **401**, 505-8.
- Wendland, B., McCaffery, J. M., Xiao, Q., and Emr, S. D. (1996). A novel fluorescence-activated cell sorter-based screen for yeast endocytosis mutants identifies a yeast homologue of mammalian eps15. *J Cell Biol* **135**, 1485-500.
- Wesche, S., Arnold, M., and Jansen, R. P. (2003). The UCS domain protein She4p binds to myosin motor domains and is essential for class I and class V myosin function. *Curr Biol* **13**, 715-24.
- Westerfield, M. (2000). "The Zebrafish Book. A guide for the laboratory use of zebrafish (*Danio rerio*)." University of Oregon Press, Eugene, OR.
- Williamson, R. A., Henry, M. D., Daniels, K. J., Hrstka, R. F., Lee, J. C., Sunada, Y., Ibraghimov-Beskrovnaya, O., and Campbell, K. P. (1997). Dystroglycan is essential for early embryonic development: disruption of Reichert's membrane in Dag1-null mice. *Hum Mol Genet* **6**, 831-41.
- Wohlgemuth, S. L., Crawford, B. D. and Pilgrim, D. B. (2007) The myosin co-chaperone UNC-45 is required for skeletal and cardiac muscle function in zebrafish. *Dev Biol* **303**, 483-92.
- Wong, K. C., Naqvi, N. I., Iino, Y., Yamamoto, M., and Balasubramanian, M. K. (2000). Fission yeast Rng3p: an UCS-domain protein that mediates myosin II assembly during cytokinesis. *J Cell Sci* **113** (Pt 13), 2421-32.
- Woods, I. G., and Talbot, W. S. (2005). The you gene encodes an EGF-CUB protein essential for Hedgehog signaling in zebrafish. *PLoS Biol* **3**, e66.
- Wray, J. S., Vibert, P. J., and Cohen, C. (1975). Diversity of cross-bridge configurations in invertebrate muscles. *Nature* **257**, 561-4.
- Xu, X., Meiler, S. E., Zhong, T. P., Mohideen, M., Crossley, D. A., Burggren, W. W., and Fishman, M. C. (2002). Cardiomyopathy in zebrafish due to mutation in an alternatively spliced exon of titin. *Nat Genet* **30**, 205-9.
- Yelon, D. (2001). Cardiac patterning and morphogenesis in zebrafish. *Dev Dyn* **222**, 552-63.
- Yelon, D., Home, S. A., and Stainier, D. Y. (1999). Restricted expression of cardiac myosin genes reveals regulated aspects of heart tube assembly in zebrafish. *Dev Biol* **214**, 23-37.

- Young, J. C., Barral, J. M., and Ulrich Hartl, F. (2003). More than folding: localized functions of cytosolic chaperones. *Trends Biochem Sci* **28**, 541-7.
- Young, J. C., Moarefi, I., and Hartl, F. U. (2001). Hsp90: a specialized but essential protein-folding tool. *J Cell Biol* **154**, 267-73.
- Yu, L. C., Dowben, R. M., and Kornacker, K. (1970). The molecular mechanism of force generation in striated muscle. *Proc Natl Acad Sci U S A* **66**, 1199-205.
- Yu, Q., and Bernstein, S.J. (2003). UCS proteins: Managing the myosin motor. *Curr Biol* **13**, R525-R527.

7. Appendices

Appendix I Primer Sequences

Primer Name	Sequence (5'-3')
Bactin FOR	GAGGCTCAGAGCAAGAGAGGT
Bactin REV	AAGCGTACAGAGAGAGCACAG
C1_GFP_FOR	AAAAAGAATTCGCTGGTTTAGTGAACCGTCAG
C1_GFP_REV	AAAAAGCGGCCGCTTATGCTTGAGCTCGAGAT CTGAGT
CS3MT UNC FOR	AAAAAAGAATTCGGGAGAAATTGGAGATTCAG
CS3MT UNC REV	TTTTTCTCGAGAGTCGTTTCAACTTTCCCAG
FOR26_full	CTTGAAGTGCTGCTACTACTA
Gibco Adapter primer	GGCCACGCGTCGACTAGTAC(T) ₁₇
REV3783_full	TGCCAGCTCATTGGATTAG
RT FOR 1	TGACGATGGGAGAAATTGG
RT REV 1	TCTCGGGTTTTCCAGTGTC
UNC_N1_FOR1	AAAAAGAATTCAACCAAACGACAAGAAACAAC
UNC_N1_REV1	AAAAAGGATCCCTGAGGGTCTCCAGAAAGGTC
ZF GCUNC45 3'RACE 2	CACGCTGGAACGCCGATGTCAA
ZF GCUNC45 3'RACE 3	GCCGATGTCAAAGAGGACCT

ZF PGEX2T FOR	GGGTTGGATCCTTGACGATGGGAGAAATTGG
ZF PGEX2T REV1	TTTTTGAATTCGGTTTTTCAGCTTTTGTTGG
ZF PGEX2T REV3	AAAAAGAATTCTCTCGGGTTTTCCAGTGTC
ZF PGEX2T REV7	AAAAAGAATTCAGTTGGAAAAGGGCTTTAT
ZF UNC45B FOR	CAGGGAGGAAGGAAACAACCA
ZF UNC45B FOR2	AAAAAGAATTCGACGAAGCTGAATTGACTT
ZF UNC45B REV	CGGCTTTACGGACAGAGGCTT
ZF UNC45B REV2	AAAAAGAATTCTCCCAGAAAGCAGACCATTC

Appendix II Nucleotide sequence of *unc45a* cDNA

ACGCGGGGGCGCATCAAAACCAGGAAGCGTACTGGCAGAACAGTCGAGAACAGAACTACTGTACA
TTTAAATAACAACAGCAATATTTTAAAGCGTTATATAACACGAATCAAATCATTAGGCTATATTAT
AAACTCTGAATCTTCTTTTATCTAAACAGCGAATACAACGCTACAGAGAAAGATGTCCCAGG
ACTCCTCTGCACTGAGGGAGGAAGGAAACAACCATTTTAAAGCCGGCGATGT
CCAACAAGCTCTGACCTGCTACACAAAAGCCCTGAAGATCAGCGACTGTCCA
TCCGAGAGTGCAGTCCTGTACCGCAACCGCTCAGCATGTTATCTTAAACTCGA
GGACTACACCAAAGCAGAAGAAGATGCCACAAAATCTTTGGATGTTGATCCA
GGTGACATCAAGGCCCGGTTTCGTCGAGCGCAGGCTCTGCAGAAGCTCGGGC
GGCTGGATCAGGCGTTCATGGACGTTCAGAAATGTGCACAGCTCGAACCCAA
GAATAAAGCTTTCCAGGATCTGCTTAGACAGCTCGGAGCTCAGATCCAGCAG
AAGGCAACACAGCTCTCCTCCACAGACTCACGGGTTCAACAGATGTTCAAAC
TCCTCCTGGATAGTTCAGCACCAATTGCAGACAGACAGAAGGCTGCTCAGAA
TCTGGTGGTACTTTCCCGAGAGGACGCTGGCGCTGAGCAGATCTTCAGGAAT
GATGGAGTCAAACCTCCTGCAGAATCTCCTTGAGTCCAAACAAGAGGAACTCA
TCCTTTCTGCTCTCCGGACTCTGGTCGGCCTGTGCACCGGACACCAGTCCAGG
ACCATGGCCATTGTGAACGAGCTGGGGATGGAGCGATTGTGTGGCGTCATGG
GCTCTTCTGCCTCCAGCGTGTCTCTTTCAGCCTGCCATCTGCTCCAGGTGATGT
TTGAGGCTCTTACAGAGGGCATGAAGAAACTGATCCGAGGGAAAGATGAAG
CCATACTGCCAGAACCGTCCCGCGAGTTGCGCTCCATGTTGCGTCATCTGCTT
GACATGCTGCCTGCATCCAGTGTGTCTGGTGCGGGACGTGACAGTGCCATCA
ACCTGCTGGTCAAACAGGTGCCCCGAAAATCAGTGAAAAACCCAGACAACTC
ACTCAGCCTGTGGGTCATTGACCAGGGGCTTAAGAAGATTCTGGAAGTTGCT

GGAAGTGTGCTGAGGTTGAAAACGGGCGCCGCTCACTGAAAACACTCACA
TGAGCTGCTCTGTGCTGCTTAATAAGCTTTATGATGACCTGAAGAGCGATAAA
GAGAGGGAGAACTTCAGCAAGCTCTGTGAGGAATACGTGCAGCATCACTTCA
TGAGTTCGTCTATGGAGCGTCGTCTGCGGGCGATCCAGACGGTCTCAGTGCTC
CTGCAGGGTCCCAGCGATGTGGGAAACGTGACTCTGGAGCTGAGCGGCCTCA
TGGACTCGGTCATCTCACTGTGTGCGTCTGAAGACATCGTCCAGCAGCAGGT
GGCAGTGGAGGCGCTCATTACGCCGCGAGGCAAAGCCAAGAGAGCCTCCTTC
ATCACTGCCAATGGTGTGGCGCTGCTCAAAGAGCTCTACAAGAAGAGCCAGA
ACGACCGCATACGAGTGC GCGCGCTGGTGGGATTGTGTAAACTGGGCTCTGC
TGGAGGCACAGATTT CAGCATGAAGCAGTTTGCTGAAGGTTCAACTCAAA
CTCGCCAAGCAGTGCAGAAAATGGCTCTGCAATGAATCTCTTCCTCCAGCA
TCGCGGCGCTGGGCCATCGAGGGTCTGGCTTACCTCACGCTGGACGCCGATG
TCAAAGAGGACCTGGTTGAGGACAAGAAAGCTCTTCAGGCCATGTTTGAGCT
GGCCAAGTCCGAGGACAAA ACTGTGTTGTTTGCGGTGGGATCGACACTGGTA
AACTGCACCAACAGCTACGATGTGGAGAAGCCAGACCCGCAGATGGTGGAG
CTAGCTAAATATGCTAAACAACATGTACCAGAGGAGCATCCAAAGGATGGCC
AGCCATTTGTGGAGCAGCGGGTGGTGAAGCTGCTGGAGGCTGGTGTGGTGTC
AGCGCTGGTCTGTATGGTGAAACAGGAGAGTCCTGCCATGACCGAGGCCTGC
AGAGAGTGCATCGCCAGGGTGATTTTGGCTCTGGTGGAGAGACAGGAGGACA
GAGGACTGGTTGTGGCACAAGGAGGAGGAAAAGCTTTGCTGCCGCTGGTTTC
TGAGAGCACAGATCGTGGGAAGATTAAAGCAGCTCAAGCTCTGGCCAAAATC
ACCATCACATCCAACCCAGAGATTGCATTTCCAGGCGAGAGGGTGTATGAGG
TGGTGAGACCCCTGGTCAGTCTTCTGGCTCTCGACTGCTCAATGCTGCAGAAC

TTTGAGGCTCTGATGGCCCTCACCAACCTGGCCGGCATCAGCGAGAGACTCA
GGCAGAAGATCATCAAGGAGAAAGCTGTGCCTAAAATCGAGGGCTACATGTT
TGAAGAGCACGACATGGTCCGCGCTGCTTCCACAGAGTGTATGTGCAATCTG
GCCTTAAGCACTGAGGTGCAGAAGCTTTATCTGGCCACTGAGAGTGACCGTC
TGAAGCTGCTGGTTCTCTACAGTGGTGAGGATGATGAGCGACTGAGGAAGGC
TGCGTCCGGCACACTGGCTGTGCTGACCGGGGAGATGCCCCGAGCTCTGCACA
CGTATCCCTGACACGACGAGCCACTGGCTGGAGATCTTGCAGGCTCTTTTGCT
CAGTGAAAGTCAGGACCTGCGGCACCGTGGAGTGGTCATCGTGATGAACAT
CATGCAGGCGGATAAAAGCTTAGCAGAGAAGCTGATGGAGAGCGAGGCGCT
GGAGATCCTGTCTGTGCTGACTAAAACAGACGATCCAAAGCAGGCCTCTGTC
CGTAAAGCCGCACAGCGCTGTTTAGATCTGGCTCTCGAGTACGGACTCATCC
GGAGCAACGAGAGCGGAGTTAATGGTAATTCCATTTAAGGCTGCAGTGTTTG
ATGGAGGCCGCAATGGTCACACACATTTAGACTGTCTAAAGAGACCAAATATTGAG
TTAATCACTTCCATTCCATTAGATGCACTCAATTTATTTACAAGCACATTCCGAGTTTGGACACCC
CTAGGCTATGATTATTCCACTGTAATGTGTAAGTATTGATCTTATGTTATTTATGCATTGGCACACTT
GTTTGATATCACGTCACCCTTTATACTCAAGTCTTAATTGCTGAATGTAAAAAAGATGTATTCT
GGTTTTATATAAACTACTTTTGTTCAAAAAAAAAAAAAAAAAAAAA

Sequence was provided by Dr. Beth Roman. Shaded region indicates morpholino-binding site. Small font indicates 5' and 3' UTR.

Appendix III Protein sequence of Unc45a

MSQDSSALREEGNNHFKAGDVQQALTCYTKALKISDCPSES AVLYRNRSA CYLK
LEDYTKAEEDATKSLD VDPGDIKARFRRAQALQKLGRLDQAFMDVQKCAQLEP
KNKAFQDLLRQLGAQIQKATQLSSTDSRVQQMFKLLLDSSAPIADRQKAAQNL
VVLSREDAGAEQIFRNDGVKLLQNLLESKQEELILSALRTL VGLCTGHQSRTMAI
VNELGMERLCGVMGSSASSVSL SACHLLQVMFEALTEGMKKLIRGKDEAILPEP
SRELRSMLRHLLDMLPASSVSGAGRDSAINLLVKQVPRKSVKNPDNSLSLWVID
QGLKKILEVAGTVAEVENG PPLTENTHMSCSVLLNKL YDDLKSDKERENFSKLC
EEYVQH HFMSSSMERRLR AIQTVSVLLQGPSDVGNVTLELSGLMDSVISLCASED
IVQQQVAVEALIHAAGKAKRAS FITANGVALLKEL YKKSQND RIRVRALVGLCK
LGSAGGTDFSMKQFAEGSTLKLAKQCRKWLCNESLPPASRRWAIEGLAYLT LDA
DVKEDLVEDKKALQAMFELAKSEDKTVLFAVGSTLVNCTNSYDVEKPDPQMVE
LAKYAKQHVPEEHPKDGQPFVEQRVVKLLEAGVVSALVCMVKQESPAMTEAC
RECIARVILALVERQEDRGLVVAQGGGKALLPLVSESTD RGKIKAAQALAKITITS
NPEIAFFGERVYEVVRPLV SLLALDCSMLQNFEALMALTNLAGISERLRQKIIKEK
AVPKIEGYMFEEHDMVRAASTE CMCNLALSTE VQKLYLATESDRLKLLVLYSGE
DDERLRKAASGTLAVLTGEMPELCTRI PDTTSHWLEILQALLSESQDLRHRGVV
IVMNIMQADKSLAEKLMESEALEILSVLTKTDDPKQASVRKAAQRCLDLALEYG
LIRSNE SGVNGNSIZ

Sequence was provided by Dr. Beth Roman.

Appendix IV Nucleotide sequence of *unc45b* cDNA

CCACGCGTCCGCGGACGCGTGCGCTCTTGAAGTGCTGCTACTACTATTGAACAAACCAAACGACAA
GAAACAACACTGAAGCACAGAACAGATTTCTCTTACGACGAAGCTGAATTGACTTCAATGACG
ATGGGAGAAATTGGAGATTTCAGTCCAGTTAAAGGAAGAAGGCAACAAGCAC
TTCCAGGCCGAGAAATCGACCAAGCCATTGACTGCTATACCAAAGCCATAA
AGACCTGCAAGAAAGAAGACAAGAAAGCATTAGCTGTTATCTATAGGAACA
GATCAGCCTGTTTCCTGAAAAAGGAAAATTACAGCAATGCAGCATCAGATGC
TACAAAAGCTATTGATGTGGATGCAGCAGACATCAAGGCTCTGTACAGAAGA
TGTCAAGCATTTGAGAAGCTCGGCAAACCTGGACATGGCCTTTAAAGACGTCC
AGAGGTGTGCAACCATCGAGCCGAAAAACAAGACCTTTCTGGAGACCCTCAG
GAGACTTGGAGCTGAAATCCAACAAAAGCTGAAAACCACATTTTCAACAGAC
TCCAGAGTGCAAAACATGTTTGACATCCTATTTTCAGACGAACCAGATAAGG
AAAAGAGAGAAAAGGCAGCCAATAACCTGATAGTCCTTGCCAGAGAGGATG
CGGGTGCAGAGAGAATCTTCCAGAACAACGGCGTGCCTTTACTCATGCAGCT
GATCGACACTGGAAAACCCGAGATGATTCTGGCTGCCATCCGGACGCTGTCA
GGAATGTGCACAGGACACCGAGCCAGGGCAACTGCCATCATTCAATTCAGTGG
GAATCAGCAAACCTGTGCAGCATCATGGCTGTGGACAATGAAGAGATTGCTCT
GGCTACTGCAAACCTCTTCCAGTGTGTCAATGACTCTCTGTCTGGAGGAGACA
AGAGAAACTACGGCAAAGAAGAGGCCCTCGTACTCGACTCTAGTAAGGACTT
GAAGGATATCCTGCTGGCCTTGTTGGAGATGATCGCAAGCAAGAACGTTTCT
GGACACGGCCGAGACCAGGCGCTCAACCTGCTAACTAAAAATGTGCCAAGA
CAAAACAAGAAAAGCACTGATAACTCCAAGTGCCTTTTCACCATTGACCACG

GCCTGAAGAAGATTCTGAAGGTGTGCGGTCAAGTTCCAGATCTTCCAGATCA
GCTGCCGATGACAGAAAACACACAGCTGATTGCCAGCGTTCTCCTCAGCAAG
CTGTATGATGACCTGCGCTGTGATCCTGAGAGGGATCAATTCAGAGATATCT
GTGATGACTATATTAAGAGCAAGTTTGACCCCAACGACATGGACAAGAACAT
CCACGCCATCAACACTCTGTCAGGTATCCTTCAGGGGCCTTTCGATCTGGGCA
ATGTCCTGGCAGGTCGGCAGGGTGTAATGGAGATGATGGTGGCCCTCTGCGG
ATCTGAGCGTGAAGTTGACCAACTGGTAGCAGTGGAGGCCCTCATTACGCC
TCCACCAAACCAGCAAAGCCTCCTTCTTCATCAGCAACGGAGTCTCTCTGCT
GAAGGAGATGTACAAGAAGACCAAGAACGAGAAGATCAAGATCAGAGCTCT
TGTGGGTCTGTGCAAACCTGGGATCCGCTGGGGGAGATGATTACAGCATGAGG
CAGTTTGCAGAGGGTTCCACAGAGAAACTGGCCAAGCAGTGCAGAAAGTGG
CTTTGCAATCCCACCCTTGACGTGCGCACCAGAAAGTGGGCCATTGAAGGTTT
GGCTTACCTCACCAATGATGCTGATGTGAAAGATGATTTTGCTGAGGATGAA
CCAGCCATGAGGGCCATGTTTGAACCTACCAAGTCAAATGATAAGACCATCC
TGTATGCGGTCGCCTGCACCCTTGTCAACTGCACCAACAGCTATGATAAGAA
AGAGATCATCCCTGAAATGGTGCAACTGGCCAAATTCTCCAAACAACACGTC
CCTGAGCAACATCCAAAGGATAAGAAGGACTTCATTGTAAGGCGAGTGAAA
AGACTATTGAAAGCTGGAGTCACGTCAGCACTCGCTGTCATGGTCAAAGCTG
ATAACTCCATCCTGACTGATCAGACCAAAGAAATGCTCGCAAGAGTGTTCTT
TGCCTTGACTGAAGATGTTAAAGATCGCGGCATAATCGTAGCTCAAGGTGGA
GGAAAGGCTTTGATCCCATTAGCCCTGGAAGGAACTGACAAAGGAAAAATA
AAAGCCAGCCATGCTCTGGCTAAGATCGCTGCTGTATCCAATCCTGAAATCG
CTTCCCTGGAGAGAGGATTTATGAAGTTGTGCGCCCACTGGTTTCCTTGCTA

GGCACAGATCGTGATGGAATGGAAAAC TTTGAAGCTCTGCGGGGTCTTACAA
ATCTTGCTGGCTTAAACGACAAACTTCGGGTAAAAATCCTGAAAGAAAAAGC
GCTGCCAGAAATCGAGAACTACATGTTTGAGGATCATGAACAGATCAGACAA
GCCGCCACAGAGTGCATGTGCAATCTAGTTTGCTGCAAGGAGGTCCAAGACA
GATATCTGGAAGACGGCAACGATAAGCTCAAGCTTCTAGTGCTTCTCTGCGG
TGAGGATGAGGAGAAGCTTCAGAGGGCAGCAGCCGGGGCTCTGGCGATGCT
CACTGCCGCACAGAAGAAACTGGCTGTTAAGATGACTAAAGTGACTGAGCAG
TGGCTTGAGATCCTGCAGAGACTGTGCATCCACGATAACCCTGAGATTCAAC
ACAGAGGTCTTGTGACGGTGTTTAACATGCTGGATGCTGATGATCAACTAGC
CAAGAAGCTCGTTGAAAGTGACATGCTGGAAATCCTGACATATGTGGCAAAA
CTAGAGGACAACCCCAAGAAGCAGAACGCTATAGACGCAGCTCGTGCCTGCC
TGTCCAAAGCCATGGATAATGGACTTATAAAGCCCTTTTCCAAGTAGACCGAAA
ATTGTGTCGTTAGGTGAAGAGAGAATGGTCTGCTTTCTGGGAAAGTTGAAACGACTGCAAACCATT
TGTAATATGTTTTTTTTTTTTTATTTGTAATGATGCGCATGGTTCCTTTTTCCAGATTGTATGTTTGTA
TGGCTGTTTGTTGATCAATGGTTTTGGATTGGTATTTATATTTTATGTATTTGCACATTTACATTTT
TGATTCATAGATTTATTTTGTTTTTTTIGAGAAGACTACTGGAATATTTTTTAATTTTACATGATCA
ATGTTTGTTTTAATGTAATACATTCTATTGCAGGGGTGCCCAAACCTCGTTCCTGGAGGGCCTGCAAA
GTTTACTTCCAACCCTAATCAGACACACCTGGGCTAGCTAATCAAGCTCTTAAGCTGCAGGGCCTTT
CCTCCCATAGACTTCTATTCATACGCATGCGAATGCGTCAGACCGGAAACGCAGGGTCATGCGTCA
AGTTTTGCAGGTGCTGCGGTGCAAAGTTCAAGCTTGGTGAACCTGACCTGCGAAATCGCATCACT
TGACTGCGTGAGACCAATTGAAGTCAAAAACATGACCTCTCTGGACAGAAGTTTAAAACATGGAGC
AATCACTCACTTTTTAAAATGTCTAATCATCTTGTTTAATTTGCCCCCTTTTTCGCAGCGCCGCATGA
CAGAAAATCGCATGCTCAAACCTCTAGTGGGACTGCACCTTTACTAGGCTTTCTAGAAACATCCGTG

GAGATGTGTTGAGTCAAGTTGGAGCTAAAAATCTGCAGGACACCAGCTCTCCAGGACCGAGTTGGGG
CACCCCCGTTCTATTGTATTGCTAAATCCAAATGAGCTGGCAATCTGTAAAAACATCCCAAATCAAT
AAATGCTATTAAGTAATTATTATTAaaaaaaaaaaaaaaaaaaaa

Shaded region indicates morpholino-binding sites. Small font indicates 5' and 3' UTR.

Appendix V Protein sequence of Unc45b

MTMGEIGDSVQLKEEGNKHFAQAGEIDQAIDCYTKAIKTCKKEDKKALAVIYRNR
SACFLKKENYSNAASDATKAIDVDAADIKALYRRCQAFEKLGKLDMAFKDVQR
CATIEPKNKTFLLETLRRLGAEIQQLKTTFTSDSRVQNMFDILFSDEPDKEKREKA
ANNLIVLAREDAGAERIFQNGVPLLMQLIDTGKPEMILAAIRTLSGMCTGHRAR
ATAIIHSVGISKLCSIMAVDNEEIALATANLFQCVNDSLSSGGDKRNYGKEEALVL
DSSKDLKDILLALLEMIASKNVSGHGRDQALNLLTKNVPRQNKKSTDNSKCLFTI
DHGLKKILKVCQGQVPDLPDQLPMTENTQLIASVLLSKLYDDLRCDPERDQFRDIC
DDYIKSKFDPNDMDKNIHAINTLSGILQGPFDLGNVLAGRQGVMMEMMVALCGSE
REVDQLVAVEALIHASTKTSKASFFISNGVSLLKEMYKKTKNEKIKIRALVGLCK
LGSAGGDDYSMRQFAEGSTEKLAKQCRKWLCNPTLDVRTRKWAIEGLAYLTND
ADVKDDFAEDEPAMRAMFELTKSNDKTILYAVACTLVNCTNSYDKKEIPEMVQ
LAKFSKQHVPEQHPKDKKDFIVRRVKRLKAGVTSALAVMVKADNSILTDQTKE
MLARVFLALTEDVKDRGIIVAQGGGKALIPLALEGTDKGKIKASHALAKIAAVSN
PEIAFPGERIYEVVRPLVSLLGTDRDGMENFEALRGLTNLAGLNDKLRVKILKEK
ALPEIENYMFEDHEQIRQAATECMCNLVCCKEVQDRYLEDGNDKLKLLVLLCGE
DEEKLQRAAAGALAMLTAAQKKLAVKMTKVTEQWLEILQRLCIHDNPEIQHRG
LVTVFNMLDADDQLAKKLVESDMLEILTYVAKLEDNPKKQNAIDAARACLSKA
MDNGLIKPFSN